GENES ENCODING NOVEL PROTEINS WITH PESTICIDAL ACTIVITY AGAINST COLEOPTERANS

CROSS-REFERENCE PARAGRAPH

This application claims the benefit of U.S. Provisional Application No. 60/242,838, filed October 24, 2000, the teachings of which are herein incorporated by reference.

FIELD OF THE INVENTION

The present invention relates to naturally occurring and recombinant nucleic acids obtained from *Bacillus thuringiensis Cry8*-like genes that encode δ-endotoxins characterized by pesticidal activity against pests of the order Coleopteran. Compositions and methods of the invention utilize the disclosed nucleic acids, and their encoded pesticidal polypetides, to control plant pests.

BACKGROUND OF THE INVENTION

Insect pests are a major factor in the loss of the world's agricultural crops. Insect pest-related crop loss from corn rootworm alone has reached one billion dollars a year. For example, corn rootworm feeding can be economically devastating to agricultural producers. The western corn rootworm is a major insect pest of corn or maize in many regions of the world. While not as important a pest as the western corn rootworm, the southern corn rootworm may occasionally cause significant economic damage to corn. Damage from western and southern corn rootworms may result in increased lodging, reduced drought tolerance and ultimately, crop yield reductions.

Traditionally, the primary methods for impacting corn rootworm populations are crop rotation and the application of broad-spectrum chemical insecticides. Unfortunately, some species of pests have developed resistance to the chemical insecticides. Furthermore, consumers and government regulators alike are becoming increasingly

concerned with the environmental hazards associated with the production and use of synthetic chemical pesticides. Because of such concerns, regulators have banned or limited the use of some of the more hazardous pesticides. Thus, there is substantial interest in developing alternative pesticides.

Biological control of insect pests of agricultural significance using a microbial agent, such as fungi, bacteria, or another species of insect affords an environmentally friendly and commercially attractive alternative. Generally speaking, the use of biopesticides presents a lower risk of pollution and environmental hazards, and they provide greater target specificity than is characteristic of traditional broad-spectrum chemical insecticides. In addition, biopesticides often cost less to produce and thus improve economic yield for a wide variety of crops.

Certain species of microorganims of the genus *Bacillus* are known to possess pesticidal activity against a broad range of insect pests including *Lepidoptera, Diptera, Coleoptera, Hemiptera*, and others. *Bacillus thuringiensis* and *Bacillus papilliae* are among the most successful biocontrol agents discovered to date. Insect pathogenicity has also been attributed to strains of: *B. larvae, B. lentimorbus, B. papilliae, B. sphaericus, B. thuringiensis* (Harwook, ed., ((1989) Bacillus Plenum Press), 306) and *B. cereus* (WO 96/10083). Pesticidal activity appears to be concentrated in parasporal crystalline protein inclusions, and several genes encoding these pesticidal proteins have been isolated and characterized (see, for example U.S. Patent No. 5,366,892).

Microbial insecticides, particularly those obtained from *Bacillus* strains, have played an important role in agriculture as alternatives to chemical pest control. Recently, agricultural scientists have developed crop plants with enhanced insect resistance by genetically engineering crop plants to produce pesticidal proteins from *Bacillus*. For example, corn and cotton plants genetically engineered to produce pesticidal proteins isolated from strains of *B. thuringiensis*, known as δ-endotoxins or Cry toxins, are now widely used in American agriculture and have provided the farmer with an environmentally friendly alternative to traditional insect-control methods. In addition, potatoes genetically engineered to contain pesticidal Cry toxins have been sold to the American farmer. However, while they have proven to be very successful commercially, these genetically engineered, insect-resistant crop plants provide resistance to only a

narrow range of the economically important insect pests. Some insects, such as Western corn rootworm, have proven to be recalcitrant, and the level of *Bt*-toxin resistance is increasing in formerly susceptible populations of some important insect pests.

Although numerous investigators have attempted to make mutant endotoxin proteins with improved insecticidal activity, few have succeeded. In fact, the majority of genetically engineered *B. thuringiensis* toxins that have been reported in the literature report endotoxin activity that is no better than that of the wild-type protein, and in many cases, the activity is decreased or destroyed altogether. Thus, new microbial insecticides having altered specificity and/or improved pesticidal activity are desired for use in pest-management strategies.

SUMMARY OF THE INVENTION

Compositions and methods are provided for impacting plant pests, particularly Coleopteran insect pests. More specifically, the invention relates to methods of impacting insects utilizing nucleic acids derived from δ-endotoxin genes to produce transformed microorganisms and plants that express a pesticidal polypeptide of the invention. The compositions and methods of the invention find use in agriculture for controlling pests of crop plants.

The invention provides nucleic acids, and fragments and variants thereof, which encode polypeptides that possess pesticidal activity against pests of the order Coleoptera. The wild-type (e.g., naturally occurring) nucleotide sequences of the invention, which were obtained from strains of *Bacillus thuringiensis,* encode Cry-8-like δ-endotoxins.

The invention further provides fragments and variants of *Cry-8* like nucleotide sequences that encode biologically active (e.g., pesticidal) polypeptides. In particular embodiments, the disclosed nucleotide sequences encode polypeptides that are pesticidal for at least one insect belonging to the order Coleopteran (e.g., Colorado potato beetle, southern corn rootworm, and western corn rootworm).

Other embodiments of the invention provide nucleic acid encoding truncated versions of a Cry8 endotoxin that are characterized by pesticidal activity that is either equivalent to, or improved, relative to the activity of the corresponding full-length

endotoxin. Some of the truncated nucleic acids of the invention can be referred to as either fragments or variants. In particular embodiments, some of the nucleic acid fragments/variants of the invention are truncated at the 3' end of a wild-type coding sequence; in alternative embodiments, other nucleic acids of the invention comprise a contiguous sequence of nucleic acid residues, derived from another coding sequence of the invention, that have been truncated at both the 5' and 3' ends.

The invention also provides recombinant *Cry8*-like nucleic acids comprising mutagenized nucleic acid sequence variants encoding *B. thuringiensis* endotoxins that have been engineered to have improved and/or altered pesticidal activities. More specifically, the invention provides mutagenized nucleic acids encoding pesticidal polypeptides that comprise an additional, or an alternative, protease-sensitive site located in domain 1 of the polypeptide variant in a region that is located between alpha-helices 3 and 4 of the encoded polypeptide.

As demonstrated herein, the presence of an additional, and/or alternative, protease-sensitive site in the amino acid sequence of the encoded polypeptide can improve the pesticidal activity and/or specificity of the variant polypeptide encoded by the nucleic acid variants of the invention. Accordingly, the *Cry8*-nucleotide sequences of the invention can be recombinantly engineered or manipulated to produce endotoxins having improved or altered activity and/or specificity compared to that of an unmodified wild-type δ-endotoxin.

For example, one type of variant nucleic acid (e.g., mutagenized *Cry8*-like nucleotide sequence) disclosed herein provides additional mutants that comprise additional codons that introduce a second trypsin-sensitive amino acid sequence (in addition to the naturally occurring trypsin site) into its encoded polypeptide. An alternative addition variant of the invention comprises additional codons designed to introduce a chymotrypsin-sensitive site located immediately 5' of the naturally occurring trypsin site.

A second alternative type of variant nucleic acid of the invention provides substitution mutants in which at least one codon of the nucleic acid that encodes the naturally occurring protease-sensitive site is destroyed, and alternative codons are introduced into the variant nucleic acid sequence in order to introduce a different (e.g.,

substitute) protease-sensitive site in its place. In a particular embodiment of this variant polynucleotide, a replacement mutant is disclosed in which the naturally occurring trypsin cleavage site present in the encoded polypeptide is destroyed and a chymotrypsin cleavage site is introduced into in its place.

It is to be recognized that any of the disclosed mutations can be engineered in any polynucleotide sequence of the invention that comprises the amino acid residues providing the trypsin cleavage site that is targeted for modification. Accordingly, variants of either full-length endotoxins or fragments thereof can be modified to contain additional or alternative cleavage sites.

The nucleic acids of the invention can be used to produce expression cassettes that can be used to produce transformed microorganisms comprising a nucleic acid of the invention. The resulting transformants can be used in the preparation of pesticidal compositions comprising a transformed microorganism, or for the production and isolation of pesticidal proteins. Thus, the invention further provides pesticidal compositions, comprising either pesticidal polypeptides or transformed microorganisms, and methods for producing such compositions. The pesticidal compositions of the invention find use in agricultural methods for impacting pests. For example, the compositions can be used in a method that involves placing an effective amount of the pesticidal composition in the environment of the pest by a procedure selected from the group consisting of spraying, dusting, broadcasting, or seed coating.

The invention further provides isolated pesticidal (e.g., insecticidal) polypeptides encoded by either a naturally occurring, or a modified (e.g., mutagenized or manipulated) nucleic acid of the invention. In particular examples, pesticidal proteins of the invention include full-length δ-endotoxin proteins, fragments of full-length δ-endotoxins, and variant polypeptides that are produced from mutagenized nucleic acids designed to introduce particular amino acid sequences into the polypeptides of the invention. In particular embodiments, the polypeptide fragments and polypeptide variants of the invention have enhanced pesticidal activity relative to the activity of the naturally occurring δ-endotoxin from which they are derived. Polypeptides of the invention can be produced either from a nucleic acid disclosed herein, or by the use of standard molecular biology techniques. For example, a truncated protein of the invention can be produced

by expression of a recombinant nucleic acid of the invention in an appropriate host cell, or alternatively by a combination of *ex vivo* procedures, such as protease digestion and purification.

The nucleic acids of the invention can also be used to produce transgenic (e.g., transformed) plants that are characterized by genomes that comprise at least one stably incorporated nucleotide construct comprising a coding sequence of the invention operably linked to a promoter that drives expression of the encoded pesticidal polypeptide. Accordingly, transformed plant cells, plant tissues, plants, and seeds thereof are also provided.

In a particular embodiment, a transformed plant of the invention can be produced using a nucleic acid that has been optimized for increased expression in a host plant. For example, one of the pesticidal polypeptides of the invention can be back-translated to produced a nucleic acid comprising codons optimized for expression in a particular host, for example a plant, more specifically for expression in a *Zea mays* plant. Expression of a coding sequence by such a transformed plant (e.g., dicot or monocot) will result in the production of a pesticidal polypeptide and confer increased insect resistance to the plant. In a particular embodiment, the invention provides transgenic plants expressing pesticidal polypeptides that find use in methods for impacting the Colorado potato beetle, western corn rootworm, and southern corn rootworm.

DETAILED DESCRIPTION OF THE INVENTION

The invention is drawn to compositions and methods for impacting pests, particularly plant pests, more specifically insect pests of the order Coleopteran. More specifically, the isolated nucleic acids of the invention, and fragments and variants thereof, comprise nucleotide sequences that encode pesticidal polypeptides (e.g., proteins). The disclosed pesticidal proteins are biologically active (e.g., pesticidal) against insect pests, particularly the Colorado potato beetle (*Leptinotarsa decemlineata),* the western corn rootworm (*Diabrotica virgifera virgifera*), and the southern corn rootworm (*Diabrotica undecimpunctata howardi*).

The compositions of the invention comprise isolated nucleic acids, and fragments and variants thereof, that encode pesticidal polypeptides, expression cassettes comprising

nucleotide sequences of the invention, isolated pesticidal proteins, and pesticidal compositions. In some embodiments, the invention provides modified Cry8-like δ-endotoxin proteins characterized by improved insecticidal activity against Coleopterans relative to the pesticidal activity of the corresponding wild-type parental protein. The invention further provides plants and microorganisms transformed with these novel nucleic acids, and methods involving the use of such nucleic acids, pesticidal compositions, and transformed organisms in impacting insect pests.

In the description that follows, a number of terms are used extensively. The following definitions are provided to facilitate understanding of the invention.

As used herein, "nucleic acid" includes reference to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues (e.g., peptide nucleic acids) having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides.

As used herein the terms "encoding" or "encoded", when used in the context of a specified nucleic acid, means that the nucleic acid comprises the requisite information to direct translation of the nucleotide sequence into a specified protein. The information by which a protein is encoded is specified by the use of codons. A nucleic acid encoding a protein may comprise non-translated sequences (e.g., introns) within translated regions of the nucleic acid, or may lack such intervening non-translated sequences (e.g., as in cDNA).

As used herein "full-length sequence" in reference to a specified polynucleotide or its encoded protein means having the entire nucleic acid sequence or the entire amino acid sequence of, a native (non-synthetic), endogenous sequence. A full-length polynucleotide encodes the full-length, catalytically active form of the specified protein.

As used herein, the term "antisense" used in the context of orientation of a nucleotide sequence refers to a duplex polynucleotide sequence that is operably linked to a promoter in an orientation where the antisense strand is transcribed. The antisense strand is sufficiently complementary to an endogenous transcription product such that translation of the endogenous transcription product is often inhibited.

The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residues is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

The terms "residue" or "amino acid residue" or "amino acid" are used interchangeably herein to refer to an amino acid that is incorporated into a protein, polypeptide, or peptide (collectively "protein"). The amino acid may be a naturally occurring amino acid and, unless otherwise limited, may encompass known analogues of natural amino acids that can function in a similar manner as naturally occurring amino acids.

As used herein the terms "isolated" and "purified" are used interchangeably to refer to nucleic acids, or polypeptides, or biologically active portion thereof, that are substantially or essentially free from components that normally accompany or interact with the nucleic acid or polypeptide as found in its naturally occurring environment. Thus, an isolated or purified nucleic acid or polypeptide is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

As used herein the term "impacting insect pests" refers to effecting changes in insect feeding, growth, and/or behavior at any stage of development, including, but not limited to, killing the insect, retarding growth, preventing reproductive capability, and the like.

As used herein the terms "pesticidal activity" and "insecticidal activity" are used synonymously to refer to activity of an organism or a substance, such as, for example, a protein, that can be measured by, but is not limited to, pest mortality, pest weight loss, pest attraction, pest repellency, and other behavioral and physical changes of a pest after feeding and exposure for an appropriate length of time. For example "pesticidal proteins" are proteins that display pesticidal activity by themselves or in combination with other proteins.

The term "pesticidally effective amount" connotes a quantity of a substance or organism that has pesticidal activity when present in the environment of a pest. For each substance or organism, the pesticidally effective amount is determined empirically for each pest affected in a specific environment. Similarly an "insecticidally effective amount" may be used to refer to an "pesticidally effective amount" when the pest is an insect pest.

As used herein the term "recombinantly engineered" connotes the utilization of recombinant DNA technology to introduce (e.g., engineer) a change in the protein structure based on an understanding of the protein's mechanism of action and a consideration of the amino acids being introduced, deleted or substituted.

As used herein the term "mutagenized nucleotide sequence" connotes a nucleotide sequence that has been mutagenized or altered to contain one or more nucleotide residues (e.g., base pair) that is not present in the corresponding wild-type sequence, and which encodes a mutant δ-endotoxin showing improved insecticidal activity.

As used herein the term "improved insecticidal activity" characterizes a δ-endotoxin of the invention that either has enhanced anti-Coleopteran pesticidal activity relative to the activity of its corresponding wild-type protein, and/or an endotoxin that is effective against either a broader range of insects, or acquires a specificity for an insect that is not susceptible to the toxicity of the wild-type protein. A finding of enhanced pesticidal activity requires a demonstration of an increase of toxicity of at least 30% against the insect target, and more preferably 35%, 40%, 45%, or 50% relative to the insecticidal activity of the wild-type endotoxin determined against the same insect.

Units, prefixes, and symbols may be denoted in their SI accepted form. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. Numeric ranges are inclusive of the numbers defining the range. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted

single-letter codes. The above-defined terms are more fully defined by reference to the specification as a whole.

The nucleotide sequences of the invention may be used to transform any organism to produce the encoded pesticidal proteins. Methods are provided that involve the use of such transformed organisms to impact or control plant pests. The invention further relates to the identification of fragments and variants of the naturally occurring coding sequence that encode biologically active pesticidal proteins. All of the nucleotide sequences of the invention find direct use in methods for impacting pests, particularly insect pests, more particularly pests of the order Coleopteran, including, for example, the Colorado potato beetle, western corn rootworm, and southern corn rootworm. Accordingly, the present invention provides new approaches for impacting insect pests that do not depend on the use of traditional, synthetic chemical insecticides. The invention involves the discovery of naturally occurring, biodegradable pesticides and the genes that encode them.

The invention further provides fragments and variants of the naturally occurring coding sequences that also encode biologically active (e.g., pesticidal) polypeptides. The nucleic acids of the invention encompass nucleic acid sequences that have been optimized for expression by the cells of a particular organism, for example nucleic acid sequences that have been back-translated using plant-preferred codons based on the amino acid sequence of a polypeptide having enhanced pesticidal activity.

The nucleotide sequences of the invention were isolated from strains of the bacterium, *Bacillus thuringiensis*. Crude lysates prepared from cultures of the strains were discovered to have pesticidal activity against Colorado potato beetle, western corn rootworm, and southern corn rootworm. Crystal proteins were isolated from cultures of the strains. The isolated crystal proteins were tested for pesticidal activity in insect feeding assays. The results of the assays revealed that the isolated crystal proteins possessed Coleopteran pesticidal activity. An effort was undertaken to identify nucleotide sequences encoding crystal proteins from the strains, and the naturally occurring coding sequences and genomic nucleic acids of the invention were discovered.

The nucleotide sequences of the isolated nucleic acids were demonstrated to encode pesticidal proteins by transforming *Escherichia coli* with such nucleotide sequences. Lysates prepared from the transformed *E. coli* had pesticidal activity against

corn rootworms and Colorado potato beetles in feeding assays, demonstrating that the isolated nucleotide sequences of the invention encode pesticidal proteins. Depending upon the characteristics of a given lysate preparation, it was recognized that the demonstration of pesticidal activity sometimes required trypsin pretreatment to activate the pesticidal proteins.

Subsequently, nucleic acid variants and fragments encoding biologically active pesticidal polypeptides were identified. Some of the encoded pesticidal proteins require protease (e.g., trypsin) activation and other proteins were observed to be biologically active (e.g., pesticidal) in the absence of activation. In some embodiments, the nucleic acid encodes a truncated version of the naturally occurring polypeptide and as such, can be classified either as a variant or a fragment. In addition, second generation nucleic acid sequences were engineered to comprise nucleotide sequences that encode Cry8-like polypeptides characterized by improved or altered pesticidal activity relative to the pesticidal activity of the naturally occurring polypeptide.

The nucleic acids of the invention comprise isolated polynucleotides, and variants and fragments thereof, that encode biologically active (e.g., pesticidal) polypeptides, including, but not limited to, the *Cry8*-like nucleotide sequences set forth in SEQ ID NOS:1, 3, 5, 7, 9, 11, 15, 17, 19, 21, 23, 27, 28, 29, 31, 33, 39, 41, 43, and 45. The nucleotide sequences disclosed herein provide two background sequences referred to herein as 1218-1 and 49PVD into which mutations are introduced. In some instances, the sequences also provide variants of two distinct clones referred to herein as 1218-1 and 1218-2. More specifically, SEQ ID NO:15 (1218-1A) represents a variant of SEQ ID NO:5, each of which represent alternative embodiments of the 1218-1 clone. In addition, SEQ ID NO:17 (1218-2A) represents a variant of SEQ ID NO:7; each of which represent alternative embodiments of the 1218-2 clone.

The polynucleotides of the invention also include any synthetic or recombinant nucleotide sequence that encodes a pesticidal polypeptide comprising the amino acid sequences set forth in SEQ ID NOS: 2, 4, 6, 8, 10, 12, 16, 18, 20, 22, 24, 30, 32, 34, 40, 42, 44, and 46.

An "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3'

ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acids can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acids in genomic DNA of the cell from which the nucleic acid is derived.

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The present invention provides isolated nucleic acids comprising nucleotide sequences which encode the amino acid sequences set forth in SEQ ID NOS: 2, 4, 6, 8, 10, 12, 16, 18, 20, 22, 24, 30, 32, 34, 40, 42, 44, and 46. In particular embodiments, the invention provides nucleic acids comprising the nucleotide sequences set forth in SEQ ID NOS:1(*Cry1218-1* CDS) and 3 (*Cry1218-2* CDS), the maize-optimized nucleic acid set forth in SEQ ID NO:9 (mo1218-1), and the native genomic sequences set forth in SEQ ID NO:27 (genomic *Cry1218-1*) and SEQ ID NO:28 (genomic *Cry 1218-2*). The coding sequence (CDS) for SEQ ID NO: 27 runs from base pair 731-4348. The CDS for SEQ ID NO: 28 runs from base pair 1254-4883. Plasmids comprising each of these five nucleic acids were deposited on May 5, 2000 and November 2, 2000 with the Patent Depository of the American Type Culture Collection (ATCC), Manassas, Virginia, and assigned Patent Deposit Nos. PTA-1821 (corresponding to SEQ ID NO:1); PTA-1817 (corresponding to SEQ ID NO:3); PTA-2635 (corresponding to SEQ ID NO:9); PTA-2634 (comprising SEQ ID NO:27); and PTA-2636 (comprising SEQ ID NO:28).

Patent Deposits PTA-1821 and PTA-1817 comprise a mixture of 2 clones, each of which contains a part of the entire coding sequence. More specifically, the deposited plasmids encode nucleic acid molecules cloned into a TA vector (Invitrogen, Carlsbad, CA) that encode two overlapping fragments of the coding sequence. The full length coding sequence can be produced using an overlapping PCR strategy. A first PCR reaction should comprise forward and reverse primers designed to correspond to the 5' and the 3' ends of the full-length coding sequence. Suitable primers for use in PCR reactions are set forth in SEQ ID NOS:35 through 38. More specifically, SEQ ID NOS:35 and 36 provide a first primer set "(a)" comprising a forward primer SEQ ID NO:35 (5' -ATGAGTCCAAATAATCAAAATG) and a reverse primer SEQ ID NO:36 (5' -CCGCTTCTAAATCTTGTTCC) for the 5' end of the coding sequence. SEQ ID

NOS:37 and 38 provide a second primer set "(b)" comprising a forward primer SEQ ID NO:37 (5' -GGAACAAGATTTAGAGG) and a reverse primer SEQ ID NO:38 (5' -CTCATCGTCTACAATCAATTCATC) for the 3' end of the coding sequence. The two DNA bands generated by the first PCR reaction performed with the above-identified primer sets should be purified and a second round of PCR, set for 7 cycles, should be performed utilizing the purified DNA isolated from the first PCR reaction in the absence of any primers. The 3' end of the nucleic acid generated by primer set (a) and the 5' end of the nucleic acid generated by primer set (b) will overlap and prime the generation of the full-length coding sequence. A third and final PCR reaction is performed to generate the full-length coding sequence. This reaction is performed using 1 μl of the second PCR reaction product and a primer set comprising SEQ ID NO:35 (forward primer of set (a)) and SEQ ID NO:39 (reverse primer of set (b)).

The above-referenced deposits (e.g., PTA-1821; PTA-1817; PTA-2635; PTA-2634; and PTA-2636) will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. These deposits were made merely as a convenience for those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112.

Of particular interest are optimized nucleotide sequences encoding the pesticidal proteins of the invention. As used herein the phrase "optimized nucleotide sequences" refers to nucleic acids that are optimized for expression in a particular organism, for example a plant. Optimized nucleotide sequences may be prepared for any organism of interest using methods known in the art. For example, SEQ ID NO:9 discloses an optimized nucleic acid sequence encoding the pesticidal protein set forth in SEQ ID NO:16 (truncated 1218-1A). More specifically, the nucleotide sequence of SEQ ID NO:9 comprising maize-preferred codons SEQ ID NO:9 was prepared by reverse-translating the amino acid sequence set forth in SEQ ID NO:16 to comprise maize-preferred codons as described by Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498. Optimized nucleotide sequence find use in increasing expression of a pesticidal protein in a plant, particularly a monocot plant, more particularly a plant of the Gramineae (Poaceae) family, most particularly a maize or corn plant.

The invention further provides isolated pesticidal (e.g., insecticidal) polypeptides encoded by either a naturally occurring, or a modified (e.g., mutagenized or truncated) nucleic acid of the invention. More specifically, the invention provides polypeptides comprising an amino acid sequence set forth in SEQ ID NOS:2, 4, 6, 8, 10, 12, 16, 18, 20, 22, 24, 30, 32, 34, 40, 42, 44, and 46 and the polypeptides encoded by a nucleic acids described herein, for example those set forth in SEQ ID NOS:1, 3, 5, 7, 9, 11, 15, 17, 19, 21, 23, 27, 28, 29, 31, 33, 39, 41, 43, and 45, and fragments and variants thereof.

In particular embodiments, pesticidal proteins of the invention provide full-length δ-endotoxin proteins, fragments of full-length δ-endotoxins, and variant polypeptides that are produced from mutagenized nucleic acids designed to introduce particular amino acid sequences into polypeptides of the invention. In particular embodiments, the amino acid sequences that are introduced into the polypeptides comprise a sequence that provides a cleavage site for an enzyme or protease.

Some of the polypeptides of the invention, for example SEQ ID NOS:2 and 4 comprise full-length δ-endotoxins; other polypeptides such as SEQ ID NOS:6, 8, 10, 16, 18, and 20 embody fragments of a full-length δ-endotoxin; and SEQ ID NOS:12, 22, 24, 30, 32, 34, 40, 42, 44, and 46 provide polypeptide variants. Some of the polypeptide fragments and variants of the invention have enhanced pesticidal activity relative to the activity of the naturally occurring δ-endotoxin from which they are derived, particularly in the absence of *in vitro* activation of the endotoxin with a protease prior to screening for activity. For example, the data presented herein in Table 1 of Example 6 indicates that the NGRS addition mutant (SEQ ID NO:12) of SEQ ID NO:16 (truncated 1218-1A endotoxin) is characterized by increased pesticidal activity against Colorado potato beetle.

SEQ ID NOS:6, 10, 16 and 20 provide polypeptides that embody truncated versions of the 1218-1 polypeptide set forth in SEQ ID NO:2. SEQ ID NO:16 provides a variant, referred to herein as 1218-1A of the polypeptide set forth in SEQ ID NO:6 and referred to herein as 1218-1. Three of the above-mentioned sequences, SEQ ID NOS: 6, 10 and 16 represent a polypeptide that is shortened (truncated) at the 3' end of the amino acid sequence set forth in SEQ ID NO:2. In contrast, the fourth polypeptide variant set forth in SEQ ID NO:20 provides a variant that is truncated at both the 5'and 3' ends of

the full-length protein set forth in SEQ ID NO:2. SEQ ID NOS: 8 and 18 (1218-2 and 1218-2A, respectively) provide polypeptides that embody truncated versions of the polypeptides set forth in SEQ ID NO:4. Each of these two polypeptides provide a protein that is truncated at the 3' end of the full-length 1218-2 polypeptide set forth in SEQ ID NO:4.

SEQ ID NOS:12, 22, 24, 40, and 44 provide a family of polypeptides that embody variants of the 1218-1A truncated polypeptides set forth in SEQ ID NO:16, thus SEQ ID NOS:12, 22, 24, 40, and 44 provide variants (or mutants) of the biologically active fragment of the Cry8-like polypeptide set forth in SEQ ID NO:2. More specifically, SEQ ID NO:12 provides a mutant, referred to herein as NGSR.N1218-1, that comprises an additional trypsin-sensitive cleavage site; SEQ ID NO:22 provides a second mutant, referred to herein as LKMS.N1218-1, that comprises a chymotrypsin-sensitive cleavage site that is not present in the wild-type 1218-1 or 1218-1A polypeptide; and SEQ ID NO:24 provides a replacement mutant, referred to herein as LKMS.R1218-1, in which an existing trypsin cleavage-site is destroyed and a chymotrypsin site is introduced in its place. SEQ ID NO:40 provides a second chymotrypsin-addition mutant, referred to herein as LRMS.N1218-1, that comprises the alternative chymotrypsin cleavage site LRMS (SEQ ID NO:48). SEQ ID NO:44 provides a second replacement or substitution mutant, referred to herein as LRMS.R1218-1, in which the native trypsin site is replaced with the chymotrypsin cleavage site LRMS.

SEQ ID NOS:30, 32, 34, 42, and 46 provide a second family of polypeptides that embody variants or mutants of the truncated polypeptide set forth in SEQ ID NO:20. Thus, SEQ ID NOS: 30, 32, 34, 42, and 46 provide variants of the pesticidal fragment of SEQ ID NO: 2 that is set forth in SEQ ID NO: 20. More specifically, SEQ ID NO:30 provides a mutant, referred to herein as NGSR.N49PVD, that comprises an additional trypsin-senstive cleavage site; SEQ ID NO: 32 provides a second mutant, referred to herein as LKMS.N49PVD, that comprises a chymotrypsin-sensitive cleavage site that is not present in the wild-type 1218-1 or 1218-1A polypeptide; and SEQ ID NO: 34 provides a replacement mutant, referred to herein as LKMS.R49PVD, in which an existing trypsin cleavage site is destroyed and a chymotrypsin site is introduced in its place. SEQ ID NO:42 provides a second chymotrypsin addition mutant, referred to

herein as LRMS.N49PVD, that comprises the alternative chymotrypsin cleavage site LRMS (SEQ ID NO:48). SEQ ID NO:46 (LRMS.R49PVD) provides a second replacement or substitution mutant in which the native trypsin site is replaced with the chymotrypsin cleavage site LRMS.

It is to be understood that the polypeptides of the invention can be produced either by expression of a nucleic acid disclosed herein, or by the use of standard molecular biology techniques. For example, a truncated protein of the invention can be produced by expression of a recombinant nucleic acid of the invention in an appropriate host cell, or alternatively by a combination of *ex vivo* procedures, such as protease digestion and purification of a purified wild-type protein.

As used herein the term "isolated" or "purified" as it is used to refer to a polypeptide of the invention means that the isolated protein is substantially free of cellular material and includes preparations of protein having less than about 30%, 20%, 10%, or 5% (by dry weight) of contaminating protein. When the protein of the invention or biologically active portion thereof is recombinantly produced, preferably culture medium represents less than about 30%, 20%, 10%, or 5% (by dry weight) of chemical precursors or non-protein-of-interest chemicals.

It is recognized that the pesticidal proteins may be oligomeric and will vary in molecular weight, number of residues, component peptides, activity against particular pests, and in other characteristics. However, by the methods set forth herein, proteins active against a variety of pests may be isolated and characterized. The pesticidal proteins of the invention can be used in combination with Bt endotoxins or other insecticidal proteins to increase insect target range. Furthermore, the use of the pesticidal proteins of the present invention in combination with Bt δ-endotoxins or other insecticidal principles of a distinct nature has particular utility for the prevention and/or management of insect resistance. Other insecticidal principles include protease inhibitors (both serine and cysteine types), lectins, α-amylase, and peroxidase.

Fragments and variants of the nucleotide and amino acid sequences and the polypeptides encoded thereby are also encompassed by the present invention. As used herein the term "fragment" refers to a portion of a nucleotide sequence of a polynucleotide or a portion of an amino acid sequence of a polypeptide of the invention.

Fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of the native protein and hence possess pesticidal activity. Thus, it is acknowledged that some of the polynucleotide and amino acid sequences of the invention can correctly be referred to as either fragments and variants. This is particularly true of truncated sequences that are biologically active.

It is to be understood that the term "fragment," as it is used to refer to nucleic acid sequences of the invention, also encompasses sequences that are useful as hybridization probes. This class of nucleotide sequences generally do not encode fragment proteins retaining biological activity. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length nucleotide sequence encoding the proteins of the invention.

A fragment of a *Cry8*-like nucleotide sequence that encodes a biologically active portion of a pesticidal protein of the invention will encode at least 15, 25, 30, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,100, or 1,200 contiguous amino acids, or up to the total number of amino acids present in a pesticidal polypeptide of the invention (for example, 1,206, 1,210, 667, 667, and 669 amino acids for SEQ ID NOS:2, 4, 6, 8, and 10, respectively). Fragments of a *Cry8*-like nucleotide sequence that are useful as hybridization probes or PCR primers generally need not encode a biologically active portion of a pesticidal protein.

Thus, a fragment of a *Cry8*-like nucleic acid may encode a biologically active portion of a pesticidal protein, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of a pesticidal protein can be prepared by isolating a portion of one of the *Cry8*-like nucleotide sequences of the invention, expressing the encoded portion of the pesticidal protein (e.g., by recombinant expression *in vitro*), and assessing the activity of the encoded portion of the pesticidal protein.

Nucleic acids that are fragments of a *Cry8*-like nucleotide sequence comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 1,000, 1,200, 1,400, 1,600, 1,800, 2,000, 2,200, 2,400, 2,600, 2,800, 3,000, 3,200, 3,400, or 3,600 nucleotides, or up to the number of nucleotides present in a *Cry8*-like nucleotide

sequence disclosed herein (for example, 3,621, 3,633, 2,003, 2,003, 2,010, and 2010 and 2022 nucleotides for SEQ ID NOS:1, 3, 5, 7, 9, 15 and 17 respectively).

For example, SEQ ID NOS: 5, 9, 15, and 19 represent fragments of SEQ ID NO:1 and SEQ ID NOS:7 and 17 represent fragments of SEQ ID NO: 3. More specifically, particular embodiments of the nucleic acids of the invention disclose fragments derived from (e.g., produced from) a first nucleic acid of the invention, wherein the fragment encodes a truncated Cry8-like endotoxin characterized by pesticidal activity. The truncated polypeptide encoded by the polynucleotide fragments of the invention are characterized by pesticidal activity that is either equivalent to, or improved, relative to the activity of the corresponding full-length polypeptide encoded by the first nucleic acid from which the fragment is derived.

In specific embodiments, some of the nucleic acid fragments of the invention are truncated at the 3' end of the wild-type coding sequence. For example, SEQ ID NOS: 5 and 15 represent fragments of SEQ ID NO: 1 that are truncated at the 3' end. In an alternative embodiment, one of the polynucleotides of the invention, SEQ ID NO: 19, comprises a nucleic acid sequence that is truncated at both the 5' and 3' end of the truncated 1218-1 and 1218-1A toxin domain encoded by SEQ ID NOS: 5 and 15, respectively.

By "variants" is intended substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the pesticidal polypeptides of the invention. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below.

Variant nucleotide sequences also include synthetically derived nucleotide sequences, such as those generated, for example, by using site-directed mutagenesis but which still encode a pesticidal protein of the invention. Generally, variants of a particular nucleotide sequence of the invention will have at least about 40%, 50%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about 98%, 99%, or

more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein using default parameters.

As used herein the term "variant protein" encompasses polypeptides that are derived from a native protein by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Accordingly, the term variant protein encompasses biologically active fragments of a native protein that comprise a sufficient number of contiguous amino acid residues to retain the biological activity of the native protein.

Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native protein, that is, pesticidal activity as described herein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a native pesticidal protein of the invention will have at least about 40%, 50%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about 98%, 99%, or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs described elsewhere herein using default parameters. A biologically active variant of a protein of the invention may differ from that protein by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

It is recognized that the nucleic acid sequence of any one of the polynucleotides of the invention can be altered or mutagenized to alter (e.g., improve) the biological activity and/or specificity of its encoded pesticidal polypeptide. For example, SEQ ID NO: 11 represents a *Cry8*-like nucleotide sequence that has been mutagenized to comprise 12 additional nucleotides (SEQ ID NO:13) that are not present in the wild-type nucleic acid sequence (SEQ ID NO: 15) that is being altered. The nucleotide sequence inserted into the coding region of SEQ ID NO: 15 was designed to encode an NGRS addition mutant that comprises an additional trypsin cleavage site (NGSR) (SEQ ID NO:14) in the amino acid sequence of the encoded polypeptide.

More specifically, the amino acid sequence set forth in SEQ ID NO:14 was introduced between amino acid 164 and 165 of the Cry8 δ-endotoxin set forth in SEQ ID NO:16. This particular amino acid sequence was chosen because it duplicates the endogenous sequence present in the naturally occurring full-length protein (SEQ ID NO:2), and creates a second protease-sensitive site. More specifically, the modification introduces a second trypsin-like site. It is well known to those of skill in the art that trypsin cleaves bonds immediately C-terminal to arginine and lysine. As demonstrated herein the recombinantly engineered protein (SEQ ID NO:12) encoded by SEQ ID NO:11 is characterized by improved activity against Coleopterans, particularly against Colorado potato beetle (see Example 6, Table 1), southern corn rootworm (see Example 7, Tables 2 through 4 and 6), and western corn rootworm (see Example 7, Table 5).

SEQ ID NO: 21 represents a *Cry8*-like nucleotide sequence that has been mutagenized to comprise 12 additional nucleotides (SEQ ID NO:25) that are not present in the wild-type endotoxin. The inserted nucleotide sequence was designed to encode an LKMS addition mutant that comprises a chymotrypsin cleavage site (LKMS) (SEQ ID NO:26) in the amino acid sequence of the encoded polypeptide. More specifically, the LKMS addition mutant (LKMS.N1218-1) comprises a nucleotide sequence insert that introduces the amino acid sequence LKMS between amino acids 160 and 161 of SEQ ID NO:6. The LKMS replacement mutant LKMS.R1218-1 comprises a polypeptide in which the amino acid sequence LKMS is introduced between amino acid 160 and 161 of SEQ ID NO:16 and the amino acids NGS are removed from amino acid positions 161-163 of SEQ ID NO:16. This modification removes a trypsin site and introduces a chymotrypsin site. Chymotrypsin cleaves bonds immediately C-terminal to Methionine.

The LRMS addition mutant (LRMS.N1218-1) and replacement mutant (LRMS.R1218-1) provide alternative embodiments of polypeptides comprising an additional or alternative chymotrypsin cleavage site, but the LRMS mutants differ in the specific amino acid sequence (SEQ ID NO: 48) and nucleotide sequence (SEQ ID NO: 47) that is used to introduce the chymotrypsin cleavage site into the nucleic acid sequence that encodes the mutant polypeptides.

SEQ ID NO: 30 (NGSR.N49PVD), SEQ ID NO: 32 (LKMS.N49PVD), SEQ ID NO: 34 (LKMS.R49PVD), SEQ ID NO: 42 (LRMS.N49PVD), and SEQ ID NO: 46

(LRMS.R49PVD) provide mutants of the truncated pesticidal polypeptide 49PVD. The amino acid sequence of 49PVD is provided in SEQ ID NO: 20. The basic design of the these polypeptides and their nomenclature follow the same pattern discussed above for the 1218-1 truncated polypeptide, and are explained more fully elsewhere herein.

It is recognized that any nucleotide sequence encoding the amino acid sequences NGSR, LKMS, or LRMS can be used and that the exact identity of the codons used to introduce any of these cleavage sites into a variant polypeptide may vary depending on the use, i.e., expression in particular plant species. It is also recognized that any of the disclosed mutations can be introduced into any polynucleotide sequence of the invention that comprises the codons for amino acid residues that provide the native trypsin cleavage site that is targeted for modification. Accordingly, variants of either full-length endotoxins or fragments thereof can be modified to contain additional or alternative cleavage sites, and these embodiments are intended to be encompassed by the scope of the invention disclosed and claimed herein.

The invention further encompasses a microorganism that is transformed with at least one nucleic acid of the invention, with an expression cassette comprising the nucleic acid, or with a vector comprising the expression cassette. Preferably, the microorganism is one that multiplies on plants. More preferably, the microorganism is a root-colonizing bacterium. An embodiment of the invention relates to an encapsulated pesticidal protein, which comprises a transformed microorganism comprising at least one pesticidal protein of the invention.

The invention provides pesticidal compositions comprising a transformed organism of the invention. Preferably the transformed microorganism is present in the pesticidal composition in a pesticidally effective amount, together with a suitable carrier. The invention also encompasses pesticidal compositions comprising an isolated protein of the invention, alone or in combination with a transformed organism of the invention and/or an encapsulated pesticidal protein of the invention, in an insecticidally effective amount, together with a suitable carrier.

The invention further provides a method of increasing insect target range by using a pesticidal protein of the invention in combination with at least one second pesticidal protein that is different from the pesticidal protein of the invention. Any pesticidal

protein known in the art can be employed in the methods of the present invention. Such pesticidal proteins include, but are not limited to, *Bt* δ-endotoxins, protease inhibitors, lectins, α-amylases, and peroxidases.

The invention also encompasses transformed or transgenic plants comprising at least one nucleotide sequence of the invention. Preferably, the plant is stably transformed with a nucleotide construct comprising at least one nucleotide sequence of the invention operably linked to a promoter that drives expression in a plant cell. As used herein, the terms "transformed plant" and "transgenic plant" refer to a plant that comprises within its genome a heterologous polynucleotide. Generally, the heterologous polynucleotide is stably integrated within the genome of a transgenic or transformed plant such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of a recombinant expression cassette.

It is to be understood that as used herein the term "transgenic" includes any cell, cell line, callus, tissue, plant part, or plant, the genotype of which has been altered by the presence of heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

As used herein, the term "plant" includes reference to whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds, and plant cells, and progeny of same. Parts of transgenic plants are to be understood within the scope of the invention to comprise, for example, plant cells, protoplasts, tissues, callus, embryos as well as flowers, stems, fruits, leaves, roots originating in transgenic plants or their progeny previously transformed with a DNA molecule of the invention and therefore consisting at least in part of transgenic cells, are also an object of the present invention.

As used herein the term "plant cell" includes, without limitation, seeds suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. The class of plants that can be used

in the methods of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants. A preferred plant is *Solanum tuberosum.* A particularly preferred plant is *Zea mays*.

While the invention does not depend on a particular biological mechanism for increasing the resistance of a plant to a plant pest, expression of the nucleotide sequences of the invention in a plant can result in the production of the pesticidal proteins of the invention and in an increase in the resistance of the plant to a plant pest. The plants of the invention find use in agriculture in methods for impacting insect pests. Certain embodiments of the invention provide transformed maize plants, which find use in methods for impacting western and southern corn rootworms. Another embodiment of the invention provides transformed potato plants, which find use in methods for impacting the Colorado potato beetle.

One of skill in the art will readily acknowledge that advances in the field of molecular biology such as site-specific and random mutagenesis, polymerase chain reaction methodologies, and protein engineering techniques provide an extensive collection of tools and protocols suitable for use to alter or engineer both the amino acid sequence and underlying genetic sequences, of proteins of agricultural interest. Thus, the Cry8-like proteins of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art.

For example, amino acid sequence variants of the pesticidal proteins can be prepared by introducing mutations into a synthetic nucleic acid (e.g, DNA molecule). Methods for mutagenesis and nucleic acid alterations are well known in the art. For example, designed changes can be introduced using an oligonucleotide-mediated site-directed mutagenesis technique. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods in Enzymol.* 154:367-382; US Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York), and the references cited therein.

The wild-type (e.g., naturally occurring) nucleotide sequences of the invention were obtained from strains of *Bacillus thuringiensis* encoding Cry8-like δ-endotoxins. It

is well known that naturally occurring δ-endotoxins are synthesized by *B. thuringiensis* sporulating cells as a proteinaceous crystalline inclusion protoxin. Upon being ingested by susceptible insect larvae, the microcrystals dissolve in the midgut, and the protoxin is transformed into a biologically active moiety by proteases characteristic of digestive enzymes located in the insect gut. The activated δ-endotoxin binds with high affinity to protein receptors on brush-border membrane vesicles. The epithelial cells lining the midgut are the primary target of the endotoxin and are rapidly destroyed as a consequence of membrane perforation resulting from the formation of gated, cation-selective channels by the toxin.

A comparison of the amino acid sequences of Cry toxins of different specificities reveals five highly conserved sequence blocks. Structurally, the δ-endotoxins comprise three distinct domains, which are, from the N- to C-termini: a cluster of seven alpha-helices implicated in pore formation, three anti-parallel beta sheets implicated in cell binding, and a beta sandwich.

The mutant Cry8 polypeptides of the present invention were generally prepared by a process that involved the steps of: obtaining a nucleic acid sequence encoding a Cry8 polypeptide; analyzing the structure of the polypeptide to identify particular "target" sites for mutagenesis of the underlying gene sequence, based on a consideration of the proposed function of the target domain in the mode of action of the endotoxin; introducing one or more mutations into the nucleic acid sequence to produce a desired change in one or more amino acid residues of the encoded polypeptide sequence, wherein the change is designed to add a protease-sensitive cleavage site to the target region or to remove the original protease-sensitive site and to add a protease-sensitive site that is sensitive to the activity of a different protease; and expressing the mutagenized nucleic acid sequence that encodes the recombinantly engineered protein of the invention in a transformed host cell under conditions effective to obtain expression of the modified Cry8 polypeptide.

Many of the δ-endotoxins are related to various degrees by similarities in their amino acid sequences and tertiary structure, and means for obtaining the crystal structures of *B. thuringiensis* endotoxins are well known. Exemplary high-resolution crystal structure solution of both the Cry3A and Cry3B polypeptides are available in the

literature. The inventors of the present invention used the solved structure of the *Cry3A* gene (Li *et al.* (1991) *Nature* 353:815-821) to produce a homology model of the Cry8 δ-endotoxin disclosed and claimed herein as SEQ ID NO:2 to gain insight into the relationship between structure and function of the endotoxin, and to design the recombinantly engineered proteins disclosed and claimed herein. A combined consideration of the published structural analyses of *B. thuringiensis* endotoxins and the reported function associated with particular structures, motifs, and the like indicates that specific regions of the endotoxin are correlated with particular functions and discrete steps of the mode of action of the protein. For example, δ-endotoxins isolated from *B. thuringiensis* are generally described as comprising three domains, a seven-helix bundle that is involved in pore formation, a three-sheet domain that has been implicated in receptor binding, and a beta-sandwich motif (Li *et al.* (1991) *Nature,* 305:815-821).

The inventors reasoned that the toxicity of Cry8-like proteins, and specifically the toxicity of the Cry8 protein, could be improved by targeting the region located between alpha helices 3 and 4 of domain 1 of the endotoxin protein. This theory was premised both on the knowledge that alpha helices 4 and 5 of domain 1 of Cry3A δ-endotoxins had been reported to insert into the lipid bilayer of cells lining the midgut of susceptible insects (Gazit *et al.*, (1998) *PNAS USA* 95:12289-12294); the inventors' knowledge of the location of trypsin and chymotrypsin cleavage cites within the amino acid sequence of the wild-type protein; and the observation reported herein that the protein encoded by 1218-1 (i.e., SEQ ID NO:2) was more active against certain Coleopterans following *in vitro* activation by trypsin or chymotrypsin treatment. Accordingly, the inventors engineered a mutant Cry8-like protein that would comprise at least one additional trypsin cleavage site in the region located between helices 3 and 4 of domain 1.

More specifically, the inventors produced mutagenized *Cry8*-like nucleotide sequences that encode mutant Cry8 endotoxins (e.g., polypeptides) that comprise either additional, or alternative protease-sensitive sites. The invention provides mutant polypeptides that have been constructed in either a 1218-1 (SEQ ID NOS:6 or 16), or a 49PVD (SEQ ID NO:20) background. It should be understood that the designation 1218-1 as used herein encompasses two embodiments (e.g., 1218-1 and 1218-1A) of the 1218-1 nucleotide and amino acid sequences presented herein. This is particularly true

in the context of the disclosed addition and replacement mutants that have been created in either the 1218-1 or 49PVD background. It is to be understood that the nomenclature used herein to refer to a mutant such as, for example the NGSR.N1218-1 mutant described contemplates mutants created in either the 1218-1 and the 1218-1A background. For the sake of consistency, the sequences presented in the sequence listing for the 1218-1 mutants embody mutants created in the 1218-1A sequences (SEQ ID NOS:15 and 16).

Generally speaking, all of the mutant polypeptides described herein are designed to comprise at least one proteolytic cleavage site located between helix 3 and 4 of domain 1 that is not present in the wild-type polypeptide. All of the mutants disclosed herein were cloned into the pET expression system, expressed in *E. coli*, and tested for pesticidal activity first against southern corn rootworm (SCRW) and then western corn rootworm (WCRW). Additionally, the 49PVD variant (SEQ ID NO:20) and the NGSR.N1218-1 mutant (SEQ ID NO:12) were tested for pesticidal activity against the Colorado potato beetle (CPB).

Briefly, the mutants provided herein include: mutants comprising a second trypsin cleavage site (i.e., NGSR (SEQ ID NO:14)) introduced into the amino acid sequence of the fragment presented in either SEQ ID NO:6 (1218-1) or SEQ ID NO:16 (1218-1A) or the fragment presented in SEQ ID NO:20 (49PVD). Mutants that comprise a chymotrypsin cleavage site comprising either the amino acid sequence LKMS (SEQ ID NO:26) or LRMS (SEQ ID NO:48) introduced in front of (e.g., directly 5' of) the trypsin cleavage site that is naturally present in the modified polypeptide sequence; and replacement mutants in which the native trypsin site that occurs in the toxin domain of the modified polypeptide is destroyed and a chymotrypsin site (e.g., LKMS or LRMS) is introduced in its place.

The 1218-1 series of mutants disclosed herein are referred to as NGSR.N1218-1, LKMS.N1218-1, LKMS.R1218-1, LRMS.N1218-1, and LRMS.R1218-1. The amino acid sequences of these mutant polypeptides are set forth in SEQ ID NOS: 12, 22, 24, 42, and 44 respectively. The invention also provides a second series of mutant polypeptides (SEQ ID NOS:30, 32, 34, 42, and 46) in which the above-described addition (trypsin or chymotrypsin cleavage sites) and replacement (a chymotrypsin cleavage site instead of

the trypsin site) mutations were introduced into the truncated polypeptide (e.g., 49PVD) set forth in SEQ ID NO: 20. This series of mutants are referred to as NGSR.N49PVD, LKMS.N49PVD, LKMS.R49PVD, LRMS.N49PVD, and LRMS.R49PVD. The amino acid sequences of each of the 49PVD mutant polypeptides are set forth in SEQ ID NOS: 30, 32, 34, 42, and 46 respectively.

The NGSR mutants disclosed herein comprise an additional trypsin-sensitive protease site in a region of the amino acid sequence that encodes domain 1 of the polypeptide. For example, the NGSR.N1218-1 mutant comprises an NGSR sequence introduced between amino acid residues 164 and 165 of the wild-type protein. This amino acid sequence provides a second trypsin-sensitive cleavage site into the mutant endotoxin encoded by SEQ ID NO:11. More specifically, the NGSR (e.g., SEQ ID NO:14) sequence duplicates the endogenous trypsin cleavage site that is present at the target location, thereby introducing a second protease-sensitive sight into the loop region located between alpha helices 3 and 4 of domain 1. Thus, the amino acid sequence of SEQ ID NO:14, beginning at residue 160, reads NGSRNGSR. In contrast, amino acid positions 160-164 of the wild-type protein comprise the sequence NGSR.

While not bound by theory, it is believed that the presence of a second protease-sensitive (e.g., trypsin or chymotrypsin) site facilitates intramolecular proteolytic cleavage by enhancing the ability of helices 4 and 5 to separate from the rest of the toxin. The effects of enhancing the ability of helices 4 and 5 to separate from the rest of the toxin would be manifest as a more efficient pore-forming process and hence confer an increase in the insecticidal activity of the toxin. Indeed, the Cry8 mutants described herein show improved toxicity towards several Coleopteran pests. The data further suggests that the presence of the second protease-sensitive site produces a polypeptide that is more amenable to activation by the digestive processes of susceptible insects.

The mutagenized *Cry8*-like nucleotide sequences of the invention may be modified so as to change about 1, 2, 3, 4, 5, 6, 8, 10, 12 or more of the amino acids present in the primary sequence of the encoded polypeptide. Alternatively even more changes from the native sequence may be introduced, such that the encoded protein may have at least about 1% or 2%, or alternatively about 3% or about 4%, or even about 5% or more of the codons altered, or otherwise modified. It should be understood that the

mutagenized *Cry8*-like nucleotide sequences of the present invention are intended to encompass biologically functional, equivalent peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded.

One of skill in the art would recognize that amino acid additions and/or substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine, and isoleucine.

Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred.

Thus, the genes and nucleotide sequences of the invention include both the naturally occurring sequences as well as mutant forms. Likewise, the proteins of the invention encompass both naturally occurring proteins as well as variations (e.g., truncated polypeptides) and modified (e.g., mutant) forms thereof. Such variants will continue to possess the desired pesticidal activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication No. 75,444.

The deletions, insertions, and substitutions of the protein sequences encompassed herein are not expected to produce radical changes in the characteristics of the protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays, such as insect-feeding assays. See, for example, Marrone *et al.* (1985) *J. Econ. Entomol.* 78:290-293 and Czapla and Lang (1990) *J. Econ. Entomol.* 83:2480-2485, herein incorporated by reference.

Variant nucleotide sequences and proteins also encompass sequences and proteins derived from a mutagenic and recombinogenic procedure such as DNA shuffling. With such a procedure, one or more different *Cry8*-like coding sequences can be manipulated to create a new pesticidal protein possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence identity and can be homologously recombined *in vitro* or *in vivo*. For example, using this approach, full-length coding sequences, sequence motifs encoding a domain of interest, or any fragment of a nucleotide sequences of the invention may be shuffled between the *Cry8*-like nucleotide sequences of the invention and corresponding portions of other known *Cry* nucleotide sequences to obtain a new gene coding for a protein with an improved property of interest.

Properties of interest include, but are not limited to, pesticidal activity per unit of pesticidal protein, protein stability, and toxicity to non-target species particularly humans, livestock, and plants and microbes that express the pesticidal polypeptides of the invention. The invention is not bound by a particular shuffling strategy, only that at least one nucleotide sequence of the invention, or part thereof, is involved in such a shuffling strategy. Shuffling may involve only nucleotide sequences disclosed herein or may additionally involve shuffling of any other nucleotide sequences known in the art including, but not limited to, GenBank Accession Nos. U04364, U04365, and U04366. Strategies for DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-10751; Stemmer (1994) *Nature* 370:389-391; Crameri *et al.* (1997) *Nature Biotech.* 15:436-438; Moore *et al.* (1997) *J. Mol. Biol.* 272:336-347; Zhang *et al. (1997) Proc. Natl. Acad. Sci. USA* 94:4504-4509; Crameri *et al.* (1998) *Nature* 391:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

The nucleotide sequences of the invention can also be used to isolate corresponding sequences from other organisms, particularly other bacteria, and more particularly other *Bacillus* strains. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences based on their sequence homology to the sequences set forth herein. Sequences isolated based on their sequence identity to the entire *Cry8*-like sequences set forth herein or to fragments thereof are encompassed by

the present invention. Such sequences include sequences that are orthologs of the disclosed sequences. By "orthologs" is intended genes derived from a common ancestral gene and which are found in different species as a result of speciation. Genes found in different species are considered orthologs when their nucleotide sequences and/or their encoded protein sequences share substantial identity as defined elsewhere herein. Functions of orthologs are often highly conserved among species.

In a PCR approach, oligonucleotide primers can be designed for use in PCR reactions to amplify corresponding DNA sequences from cDNA or genomic DNA extracted from any organism of interest. Methods for designing PCR primers and PCR cloning are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). See also Innis *et al.*, eds. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York); Innis and Gelfand, eds. (1995) *PCR Strategies* (Academic Press, New York); and Innis and Gelfand, eds. (1999) *PCR Methods Manual* (Academic Press, New York). Known methods of PCR include, but are not limited to, methods using paired primers, nested primers, single specific primers, degenerate primers, gene-specific primers, vector-specific primers, partially-mismatched primers, and the like.

In hybridization techniques, all or part of a known nucleotide sequence is used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ^{32}P, or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the *Cry8*-like sequences of the invention. Methods for preparation of probes for hybridization and for construction of cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

For example, an entire *Cry8*-like sequence disclosed herein, or one or more portions thereof, may be used as a probe capable of specifically hybridizing to

corresponding *Cry8*-like sequences and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique among *Cry8*-like sequences and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Such probes may be used to amplify corresponding *Cry8*-like sequences from a chosen organism by PCR. This technique may be used to isolate additional coding sequences from a desired organism or as a diagnostic assay to determine the presence of coding sequences in a an organism. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Hybridization of such sequences may be carried out under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash

in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. The duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284: $T_m = 81.5°C + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\%\ form) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq$90% identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes,* Part I, Chapter 2 (Elsevier, New York); and Ausubel *et al.*, eds. (1995) *Current Protocols in*

Molecular Biology, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). Thus, isolated sequences that encode a *Cry8*-like protein of the invention and hybridize under stringent conditions to the *Cry8*-like sequences disclosed herein, or to fragments thereof, are encompassed by the present invention.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) CABIOS 4:11-17; the local homology algorithm of Smith *et al.* (1981) *Adv. Appl. Math.* 2:482; the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453; the search-for-similarity-method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* 85:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci.*

USA 87:2264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci.* USA 90:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al.* (1988) *Gene* 73:237-244 (1988); Higgins *et al.* (1989) CABIOS 5:151-153; Corpet *et al.* (1988) Nucleic Acids Res. 16:10881-90; Huang *et al.* (1992) CABIOS 8:155-65; and Pearson *et al.* (1994) *Meth. Mol. Biol.* 24:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) supra. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul *et al* (1990) *J. Mol. Biol.* 215:403 are based on the algorithm of Karlin and Altschul (1990) supra. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, word length = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul *et al.* (1997) supra. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See http://www.ncbi.hlm.nih.gov. Alignment may also be performed manually by inspection.

Unless otherwise stated, nucleotide sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 using the following parameters:

% identity using GAP Weight of 50 and Length Weight of 3; % similarity using Gap Weight of 12 and Length Weight of 4, or any equivalent program. For amino acid sequences, amino acid sequence identity values provided herein refer to the value obtained using GAP Version 10 using the following parameters: % identity using GAP Weight of 8 and Length Weight of 2, or any equivalent program. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by the preferred program.

GAP uses the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap extension penalty values in Version 10 of the Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar.

Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci.* USA 89:10915).

For purposes of the present invention, comparison of nucleotide or protein sequences for determination of percent sequence identity to the *Cry8*-like sequences disclosed herein is preferably made using the GAP program in the Wisconsin Genetics Software Package (Version 8 or later) or any equivalent program. For GAP analyses of nucleotide sequences, a GAP Weight of 50 and a Length of 3 was used.

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may

comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

(e)(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, preferably at least 80%, more preferably at least 90%, and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, more preferably at least 70%, 80%, 90%, and most preferably at least 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1°C to about 20°C, depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

(e)(ii) The term "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with at least 70% sequence identity to a reference

sequence, preferably 80%, more preferably 85%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified comparison window. Preferably, optimal alignment is conducted using the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453. An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. Peptides that are "substantially similar" share sequences as noted above except that residue positions that are not identical may differ by conservative amino acid changes.

The use of the term "nucleotide constructs" herein is not intended to limit the present invention to nucleotide constructs comprising DNA. Those of ordinary skill in the art will recognize that nucleotide constructs, particularly polynucleotides and oligonucleotides, comprised of ribonucleotides and combinations of ribonucleotides and deoxyribonucleotides may also be employed in the methods disclosed herein. The nucleotide constructs, nucleic acid, and nucleotide sequences of the invention additionally encompass all complementary forms of such constructs, molecules, and sequences. Further, the nucleotide constructs, nucleotide molecules, and nucleotide sequences of the present invention encompass all nucleotide constructs, molecules, and sequences which can be employed in the methods of the present invention for transforming plants including, but not limited to, those comprised of deoxyribonucleotides, ribonucleotides, and combinations thereof. Such deoxyribonucleotides and ribonucleotides include both naturally occurring molecules and synthetic analogues. The nucleotide constructs, nucleic acids, and nucleotide sequences of the invention also encompass all forms of nucleotide constructs including, but not limited to, single-stranded forms, double-stranded forms, hairpins, stem-and-loop structures, and the like.

A further embodiment of the invention relates to a transformed organism, preferably a transformed organism selected from the group consisting of plant and insect cells, bacteria, yeast, baculoviruses, protozoa, nematodes, and algae, comprising a DNA molecule of the invention, an expression cassette comprising the said DNA molecule, or

a vector comprising the said expression cassette, preferably stably incorporated into the genome of the transformed organism.

The *Cry8*-like sequences of the invention are provided in expression cassettes for expression in the organism of interest. The cassette will include 5' and 3' regulatory sequences operably linked to a *Cry8*-like sequence of the invention. By "operably linked" is intended a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes.

Such an expression cassette is provided with a plurality of restriction sites for insertion of the *Cry8*-like sequence to be under the transcriptional regulation of the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The expression cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a *Cry8*-like DNA sequence of the invention, and a transcriptional and translational termination region functional in the organism serving as a host. The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the host organism. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By "foreign" is intended that the transcriptional initiation region is not found in the native organism into which the transcriptional initiation region is introduced. As used herein, a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

The termination region may be native with the transcriptional initiation region, may be native with the operably linked DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot

(1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; and Joshi *et al.* (1987) *Nucleic Acid Res.* 15:9627-9639.

Where appropriate, a nucleic acid may be optimized for increased expression in the host organism. Thus, where the host organism is a plant, the synthetic nucleic acids can be synthesized using plant-preferred codons for improved expression. See, for example, Campbell and Gowri (1990) *Plant Physiol.* 92:1-11 for a discussion of host-preferred codon usage. For example, although nucleic acid sequences of the present invention may be expressed in both monocotyledonous and dicotyledonous plant species, sequences can be modified to account for the specific codon preferences and GC content preferences of monocotyledons or dicotyledons as these preferences have been shown to differ (Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498). Thus, the maize-preferred codon for a particular amino acid may be derived from known gene sequences from maize. Maize codon usage for 28 genes from maize plants are listed in Table 4 of Murray *et al.*, supra. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, and 5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498; herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. By "host cell" is meant a cell which contains a vector and supports the replication and/or expression of the expression vector. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells. Preferably, host cells are monocotyledonous or dicotyledenous plant cells. A particularly preferred monocotolydenous host cell is a maize host cell. When possible, the sequence is modified to avoid predicted hairpin secondary mRNA structures.

The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Gallie *et al.* (1995) *Gene* 165(2):233-238), MDMV leader (Maize Dwarf Mosaic Virus) (*Virology* 154:9-20), and human immunoglobulin heavy-chain binding protein (BiP) (Macejak *et al.* (1991) *Nature* 353:90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling *et al.* (1987) *Nature* 325:622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) in *Molecular Biology of RNA*, ed. Cech (Liss, New York), pp. 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology* 81:382-385). See also, Della-Cioppa *et al.* (1987) *Plant Physiol. 84*:965-968. Other methods known to enhance translation can also be utilized, for example, introns, and the like.

In preparing the expression cassette, the various DNA fragments may be manipulated so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

A number of promoters can be used in the practice of the invention. The promoters can be selected based on the desired outcome. The nucleic acids can be combined with constitutive, tissue-preferred, inducible, or other promoters for expression in the host organism. Suitable constitutive promoters for use in a plant host cell include, for example, the core promoter of the Rsyn7 promoter and other constitutive promoters disclosed in WO 99/43838 and U.S. Patent No. 6,072,050; the core CaMV 35S promoter (Odell *et al.* (1985) *Nature* 313:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell* 2:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol.* 12:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet.* 81:581-588); MAS (Velten *et al.* (1984) *EMBO J.* 3:2723-2730); ALS

promoter (U.S. Patent No. 5,659,026), and the like. Other constitutive promoters include, for example, those discussed in U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142; and 6,177,611.

Depending on the desired outcome, it may be beneficial to express the gene from an inducible promoter. Of particular interest for regulating the expression of the nucleotide sequences of the present invention in plants are wound-inducible promoters. Such wound-inducible promoters, may respond to damage caused by insect feeding, and include potato proteinase inhibitor (pin II) gene (Ryan (1990) *Ann. Rev. Phytopath.* 28:425-449; Duan *et al.* (1996) *Nature Biotechnology* 14:494-498); wun1 and wun2, US Patent No. 5,428,148; win1 and win2 (Stanford *et al.* (1989) *Mol. Gen. Genet.* 215:200-208); systemin (McGurl *et al.* (1992) *Science* 225:1570-1573); WIP1 (Rohmeier *et al.* (1993) *Plant Mol. Biol. 22*:783-792; Eckelkamp *et al.* (1993) *FEBS Letters* 323:73-76); MPI gene (Corderok *et al.* (1994) *Plant J.* 6(2):141-150); and the like, herein incorporated by reference.

Additionally, pathogen-inducible promoters may be employed in the methods and nucleotide constructs of the present invention. Such pathogen-inducible promoters include those from pathogenesis-related proteins (PR proteins), which are induced following infection by a pathogen; e.g., PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. See, for example, Redolfi *et al.* (1983) *Neth. J. Plant Pathol.* 89:245-254; Uknes *et al.* (1992) *Plant Cell* 4:645-656; and Van Loon (1985) *Plant Mol. Virol.* 4:111-116. See also WO 99/43819, herein incorporated by reference.

Of interest are promoters that are expressed locally at or near the site of pathogen infection. See, for example, Marineau *et al.* (1987) *Plant Mol. Biol.* 9:335-342; Matton *et al.* (1989) *Molecular Plant-Microbe Interactions* 2:325-331; Somsisch *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:2427-2430; Somsisch *et al.* (1988) *Mol. Gen. Genet.* 2:93-98; and Yang (1996) *Proc. Natl. Acad. Sci. USA* 93:14972-14977. See also, Chen *et al.* (1996) *Plant J.* 10:955-966; Zhang *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:2507-2511; Warner *et al.* (1993) *Plant J.* 3:191-201; Siebertz *et al.* (1989) *Plant Cell* 1:961-968; U.S. Patent No. 5,750,386 (nematode-inducible); and the references cited therein. Of particular interest is the inducible promoter for the maize PRms gene, whose

expression is induced by the pathogen *Fusarium moniliforme* (see, for example, Cordero *et al.* (1992) *Physiol. Mol. Plant Path.* 41:189-200).

Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemical-inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:10421-10425 and McNellis *et al.* (1998) *Plant J.* 14(2):247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet.* 227:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

Tissue-preferred promoters can be utilized to target enhanced pesticidal protein expression within a particular plant tissue. Tissue-preferred promoters include those discussed in Yamamoto *et al.* (1997) *Plant J.* 12(2)255-265; Kawamata *et al.* (1997) *Plant Cell Physiol.* 38(7):792-803; Hansen *et al.* (1997) *Mol. Gen Genet.* 254(3):337-343; Russell *et al.* (1997) *Transgenic Res.* 6(2):157-168; Rinehart *et al.* (1996) *Plant Physiol.* 112(3):1331-1341; Van Camp *et al.* (1996) *Plant Physiol.* 112(2):525-535; Canevascini *et al.* (1996) *Plant Physiol.* 112(2):513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Lam (1994) *Results Probl. Cell Differ.* 20:181-196; Orozco *et al.* (1993) *Plant Mol Biol.* 23(6):1129-1138; Matsuoka *et al.* (1993) *Proc Natl. Acad. Sci. USA* 90(20):9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J.* 4(3):495-505. Such promoters can be modified, if necessary, for weak expression.

Leaf-specific promoters are known in the art. See, for example, Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kwon *et al.* (1994) *Plant Physiol.* 105:357-67; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Gotor *et al.* (1993) *Plant J.* 3:509-18;

Orozco *et al.* (1993) *Plant Mol. Biol.* 23(6):1129-1138; and Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90(20):9586-9590.

Root-specific promoters are known and can be selected from the many available from the literature or isolated de novo from various compatible species. See, for example, Hire *et al.* (1992) *Plant Mol. Biol.* 20(2):207-218 (soybean root-specific glutamine synthetase gene); Keller and Baumgartner (1991) *Plant Cell* 3(10):1051-1061 (root-specific control element in the GRP 1.8 gene of French bean); Sanger *et al.* (1990) *Plant Mol. Biol.* 14(3):433-443 (root-specific promoter of the mannopine synthase (MAS) gene of *Agrobacterium tumefaciens*); and Miao *et al.* (1991) *Plant Cell* 3(1):11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean). See also Bogusz *et al.* (1990) *Plant Cell* 2(7):633-641, where two root-specific promoters isolated from hemoglobin genes from the nitrogen-fixing nonlegume *andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa* are described. The promoters of these genes were linked to a β-glucuronidase reporter gene and introduced into both the nonlegume *Nicotiana tabacum* and the legume *Lotus corniculatus*, and in both instances root-specific promoter activity was preserved. Leach and Aoyagi (1991) describe their analysis of the promoters of the highly expressed rolC and rolD root-inducing genes of *Agrobacterium rhizogenes* (see *Plant Science* (Limerick) 79(1):69-76). They concluded that enhancer and tissue-preferred DNA determinants are dissociated in those promoters. Teeri *et al.* (1989) used gene fusion to lacZ to show that the *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and that the TR2' gene is root specific in the intact plant and stimulated by wounding in leaf tissue, an especially desirable combination of characteristics for use with an insecticidal or larvicidal gene (see *EMBO J.* 8(2):343-350). The TR1' gene, fused to *nptII* (neomycin phosphotransferase II) showed similar characteristics. Additional root-preferred promoters include the VfENOD-GRP3 gene promoter (Kuster *et al.* (1995) *Plant Mol. Biol.* 29(4):759-772); and rolB promoter (Capana *et al.* (1994) *Plant Mol. Biol.* 25(4):681-691. See also U.S. Patent Nos. 5,837,876; 5,750,386; 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

"Seed-preferred" promoters include both "seed-specific" promoters (those promoters active during seed development such as promoters of seed storage proteins) as well as "seed-germinating" promoters (those promoters active during seed germination). See Thompson *et al.* (1989) *BioEssays* 10:108, herein incorporated by reference. Such seed-preferred promoters include, but are not limited to, Cim1 (cytokinin-induced message); cZ19B1 (maize 19 kDa zein); milps (myo-inositol-1-phosphate synthase); and celA (cellulose synthase) (see WO 00/11177, herein incorporated by reference). Gama-zein is a preferred endosperm-specific promoter. Glob-1 is a preferred embryo-specific promoter. For dicots, seed-specific promoters include, but are not limited to, bean β-phaseolin, napin, β-conglycinin, soybean lectin, cruciferin, and the like. For monocots, seed-specific promoters include, but are not limited to, maize 15 kDa zein, 22 kDa zein, 27 kDa zein, g-zein, waxy, shrunken 1, shrunken 2, globulin 1, etc. See also WO 00/12733, where seed-preferred promoters from *end1 and end2* genes are disclosed; herein incorporated by reference.

Where low level expression is desired, weak promoters will be used. Generally, by "weak promoter" is intended a promoter that drives expression of a coding sequence at a low level. By low level is intended at levels of about 1/1000 transcripts to about 1/100,000 transcripts to about 1/500,000 transcripts. Alternatively, it is recognized that weak promoters also encompasses promoters that are expressed in only a few cells and not in others to give a total low level of expression. Where a promoter is expressed at unacceptably high levels, portions of the promoter sequence can be deleted or modified to decrease expression levels.

Such weak constitutive promoters include, for example the core promoter of the Rsyn7 promoter (WO 99/43838 and U.S. Patent No. 6,072,050), the core 35S CaMV promoter, and the like. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; 5,608,142; and 6,177,611; herein incorporated by reference.

Generally, the expression cassette will comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Marker genes include genes encoding antibiotic resistance, such as those encoding neomycin phosphotransferase II (NEO) and hygromycin

phosphotransferase (HPT), as well as genes conferring resistance to herbicidal compounds, such as glufosinate ammonium, bromoxynil, imidazolinones, and 2,4-dichlorophenoxyacetate (2,4-D). See generally, Yarranton (1992) *Curr. Opin. Biotech.* 3:506-511; Christopherson *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6314-6318; Yao *et al.* (1992) *Cell* 71:63-72; Reznikoff (1992) *Mol. Microbiol.* 6:2419-2422; Barkley *et al.* (1980) in *The Operon*, pp. 177-220; Hu *et al.* (1987) *Cell* 48:555-566; Brown *et al.* (1987) *Cell* 49:603-612; Figge *et al.* (1988) *Cell* 52:713-722; Deuschle *et al.* (1989) *Proc. Natl. Acad. Aci. USA* 86:5400-5404; Fuerst *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:2549-2553; Deuschle *et al.* (1990) *Science* 248:480-483; Gossen (1993) Ph.D. Thesis, University of Heidelberg; Reines *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:1917-1921; Labow *et al.* (1990) *Mol. Cell. Biol.* 10:3343-3356; Zambretti *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:3952-3956; Baim *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:5072-5076; Wyborski *et al.* (1991) *Nucleic Acids Res.* 19:4647-4653; Hillenand-Wissman (1989) *Topics Mol. Struc. Biol.* 10:143-162; Degenkolb *et al.* (1991) *Antimicrob. Agents Chemother.* 35:1591-1595; Kleinschnidt *et al.* (1988) *Biochemistry* 27:1094-1104; Bonin (1993) Ph.D. Thesis, University of Heidelberg; Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Oliva *et al.* (1992) *Antimicrob. Agents Chemother.* 36:913-919; Hlavka *et al.* (1985) *Handbook of Experimental Pharmacology*, Vol. 78 (Springer-Verlag, Berlin); Gill *et al.* (1988) *Nature* 334:721-724. Such disclosures are herein incorporated by reference.

The above list of selectable marker genes is not meant to be limiting. Any selectable marker gene can be used in the present invention.

Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium*-mediated transformation (Townsend *et al.*, U.S. Patent No. 5,563,055; Zhao *et al.*, U.S. Patent No. 5,981,840), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.*, U.S. Patent No. 5,879,918; Tomes *et al.*, U.S. Patent No. 5,886,244; Bidney

et al., U.S. Patent No. 5,932,782; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment, " in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology* 6:923-926); and Lecl transformation (WO 00/28058). For potato transformation see Tu *et al.* (1998) *Plant Molecular Biology* 37:829-838 and Chong *et al.* (2000) *Transgenic Research* 9:71-78. Additional transformation procedures can be found in Weissinger *et al.* (1988) *Ann. Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou *et al.* (1988) *Plant Physiol.* 87:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology* 6:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet.* 96:319-324 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); Tomes, U.S. Patent No. 5,240,855; Buising *et al.*, U.S. Patent Nos. 5,322,783 and 5,324,646; Tomes *et al. (1995)* "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag, Berlin) (maize); Klein *et al.* (1988) *Plant Physiol.* 91:440-444 (maize); Fromm *et al.* (1990) *Biotechnology* 8:833-839 (maize); Hooykaas-Van Slogteren *et al.* (1984) *Nature (London)* 311:763-764; Bowen *et al.*, U.S. Patent No. 5,736,369 (cereals); Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues,* ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports* 9:415-418 and Kaeppler *et al.* (1992) *Theor. Appl. Genet.* 84:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports* 12:250-255 and Christou and Ford (1995) *Annals of Botany* 75:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology* 14:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed

strain or different strains, and the resulting hybrid having constitutive or inducible expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved.

The nucleotide sequences of the invention may be provided to the plant by contacting the plant with a virus or viral nucleic acids. Generally, such methods involve incorporating the nucleotide construct of interest within a viral DNA or RNA molecule. It is recognized that the recombinant proteins of the invention may be initially synthesized as part of a viral polyprotein, which later may be processed by proteolysis *in vivo* or *in vitro* to produce the desired pesticidal protein. It is also recognized that such a viral polyprotein, comprising at least a portion of the amino acid sequence of a pesticidal protein of the invention, may have the desired pesticidal activity. Such viral polyproteins and the nucleotide sequences that encode for them are encompassed by the present invention. Methods for providing plants with nucleotide constructs and producing the encoded proteins in the plants, which involve viral DNA or RNA molecules are known in the art. See, for example, U.S. Patent Nos. 5,889,191, 5,889,190, 5,866,785, 5,589,367 and 5,316,931; herein incorporated by reference.

The invention further relates to plant propagating material of a transformed plant of the invention including, but not limited to, seeds, tubers, corms, bulbs, leaves, and cuttings of roots and shoots.

The present invention may be used for transformation of any plant species, including, but not limited to, monocots and dicots. Examples of plants of interest include, but are not limited to, corn (*Zea mays*), *Brassica* sp. (e.g., *B. napus, B. rapa, B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor, Sorghum vulgare*), millet (e.g., pearl millet (*Pennisetum glaucum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), finger millet (*Eleusine coracana*)), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense, Gossypium hirsutum*), sweet potato (*Ipomoea batatus*),

cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), sugarcane (*Saccharum* spp.), oats, barley, vegetables, ornamentals, and conifers.

Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuca sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Lathyrus* spp.), and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petunias (*Petunia hybrida*), carnation (*Dianthus caryophyllus*), poinsettia (*Euphorbia pulcherrima*), and chrysanthemum. Conifers that may be employed in practicing the present invention include, for example, pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*). Preferably, plants of the present invention are crop plants (for example, corn, alfalfa, sunflower, *Brassica*, soybean, cotton, safflower, peanut, sorghum, wheat, millet, tobacco, etc.), more preferably corn and soybean plants, yet more preferably corn plants.

Plants of particular interest include grain plants that provide seeds of interest, oil-seed plants, and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum, rye, millet, etc. Oil-seed plants include cotton, soybean, safflower, sunflower, *Brassica*, maize, alfalfa, palm, coconut, flax, castor, olive etc. Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, etc.

Before plant propagation material (fruit, tuber, bulb, corm, grains, seed), but especially seed, is sold as a commercial product, it is customarily treated with a protectant coating comprising herbicides, insecticides, fungicides, bactericides, nematicides, molluscicides, or mixtures of several of these preparations, if desired together with further carriers, surfactants, or application-promoting adjuvants customarily employed in the art of formulation to provide protection against damage caused by bacterial, fungal, or animal pests. In order to treat the seed, the protectant coating may be applied to the seeds either by impregnating the tubers or grains with a liquid formulation or by coating them with a combined wet or dry formulation. In addition, in special cases, other methods of application to plants are possible, e.g., treatment directed at the buds or the fruit.

The plant seed of the invention comprising a DNA molecule comprising a nucleotide sequence encoding a pesticidal protein of the invention may be treated with a seed protectant coating comprising a seed treatment compound, such as, for example, captan, carboxin, thiram, methalaxyl, pirimiphos-methyl, and others that are commonly used in seed treatment. In one embodiment within the scope of the invention, a seed protectant coating comprising a pesticidal composition of the invention is used alone or in combination with one of the seed protectant coatings customarily used in seed treatment.

It is recognized that the genes encoding the pesticidal proteins can be used to transform insect pathogenic organisms. Such organisms include Baculoviruses, fungi, protozoa, bacteria, and nematodes.

A gene encoding a pesticidal protein of the invention may be introduced via a suitable vector into a microbial host, and said host applied to the environment, or to plants or animals. The term "introduced" in the context of inserting a nucleic acid into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

Microorganism hosts that are known to occupy the "phytosphere" (phylloplane, phyllosphere, rhizosphere, and/or rhizoplana) of one or more crops of interest may be selected. These microorganisms are selected so as to be capable of successfully competing in the particular environment with the wild-type microorganisms, provide for stable maintenance and expression of the gene expressing the pesticidal protein, and desirably, provide for improved protection of the pesticide from environmental degradation and inactivation.

Such microorganisms include bacteria, algae, and fungi. Of particular interest are microorganisms such as bacteria, e.g., *Pseudomonas, Erwinia, Serratia, Klebsiella, Xanthomonas, Streptomyces, Rhizobium, Rhodopseudomonas, Methylius, Agrobacterium, Acetobacter, Lactobacillus, Arthrobacter, Azotobacter, Leuconostoc,* and *Alcaligenes*, fungi, particularly yeast, e.g., *Saccharomyces, Cryptococcus, Kluyveromyces, Sporobolomyces, Rhodotorula,* and *Aureobasidium.* Of particular interest are such phytosphere bacterial species as *Pseudomonas syringae, Pseudomonas fluorescens, Serratia marcescens, Acetobacter xylinum, Agrobacteria, Rhodopseudomonas spheroides, Xanthomonas campestris, Rhizobium melioti, Alcaligenes entrophus, Clavibacter xyli and Azotobacter vinlandir* and phytosphere yeast species such as *Rhodotorula rubra, R. glutinis, R. marina, R. aurantiaca, Cryptococcus albidus, C. diffluens, C. laurentii, Saccharomyces rosei, S. pretoriensis, S. cerevisiae, Sporobolomyces rosues, S. odorus, Kluyveromyces veronae,* and *Aureobasidium pollulans*. Of particular interest are the pigmented microorganisms.

A number of ways are available for introducing a gene expressing the pesticidal protein into the microorganism host under conditions that allow for stable maintenance and expression of the gene. For example, expression cassettes can be constructed which include the nucleotide constructs of interest operably linked with the transcriptional and translational regulatory signals for expression of the nucleotide constructs, and a nucleotide sequence homologous with a sequence in the host organism, whereby integration will occur, and/or a replication system that is functional in the host, whereby integration or stable maintenance will occur.

Transcriptional and translational regulatory signals include, but are not limited to, promoters, transcriptional initiation start sites, operators, activators, enhancers, other

regulatory elements, ribosomal binding sites, an initiation codon, termination signals, and the like. See, for example, U.S. Patent Nos. 5,039,523 and 4,853,331; EPO 0480762A2; Sambrook *et al.* (1992) Molecular Cloning: A Laboratory Manual, ed. Maniatis *et al.* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Davis *et al.*, eds. (1980) Advanced Bacterial Genetics (Cold Spring Harbor Laboratory Press), Cold Spring Harbor, NewYork; and the references cited therein.

Suitable host cells, where the pesticidal protein-containing cells will be treated to prolong the activity of the pesticidal proteins in the cell when the treated cell is applied to the environment of the target pest(s), may include either prokaryotes or eukaryotes, normally being limited to those cells that do not produce substances toxic to higher organisms, such as mammals. However, organisms that produce substances toxic to higher organisms could be used, where the toxin is unstable or the level of application sufficiently low as to avoid any possibility of toxicity to a mammalian host. As hosts, of particular interest will be the prokaryotes and the lower eukaryotes, such as fungi. Illustrative prokaryotes, both Gram-negative and gram-positive, include *Enterobacteriaceae*, such as *Escherichia*, *Erwinia*, *Shigella*, *Salmonella*, *and Proteus*; *Bacillaceae*; *Rhizobiceae*, such as *Rhizobium*; *Spirillaceae*, such as photobacterium, *Zymomonas*, *Serratia*, *Aeromonas*, *Vibrio*, *Desulfovibrio*, *Spirillum; Lactobacillaceae*; *Pseudomonadaceae*, such as *Pseudomonas* and *Acetobacter*; *Azotobacteraceae* and *Nitrobacteraceae.* Among eukaryotes are fungi, such as *Phycomycetes* and *Ascomycetes*, which includes yeast, such as *Saccharomyces* and *Schizosaccharomyces*; and *Basidiomycetes* yeast, such as *Rhodotorula*, *Aureobasidium*, *Sporobolomyces*, and the like.

Characteristics of particular interest in selecting a host cell for purposes of pesticidal protein production include ease of introducing the pesticidal protein gene into the host, availability of expression systems, efficiency of expression, stability of the protein in the host, and the presence of auxiliary genetic capabilities. Characteristics of interest for use as a pesticide microcapsule include protective qualities for the pesticide, such as thick cell walls, pigmentation, and intracellular packaging or formation of inclusion bodies; leaf affinity; lack of mammalian toxicity; attractiveness to pests for ingestion; ease of killing and fixing without damage to the toxin; and the like. Other

considerations include ease of formulation and handling, economics, storage stability, and the like.

Host organisms of particular interest include yeast, such as *Rhodotorula sp.*, *Aureobasidium sp.*, *Saccharomyces sp.*, and *Sporobolomyces sp.*, phylloplane organisms such as *Pseudomonas sp.*, *Erwinia Sp.*, and *Flavobacterium sp.*, and other such organisms, including *Pseudomonas aeurginosa*, *Pseudomonas fluorescens*, *Saccharomyces cerevisiae*, *Bacillus thuringiensis*, *Escherichia coli*, *Bacillus subtilis*, and the like.

Genes encoding the pesticidal proteins of the invention can be introduced into microorganisms that multiply on plants (epiphytes) to deliver pesticidal proteins to potential target pests. Epiphytes, for example, can be gram-positive or gram-negative bacteria.

Root-colonizing bacteria, for example, can be isolated from the plant of interest by methods known in the art. Specifically, a *Bacillus cereus* strain that colonizes roots can be isolated from roots of a plant (see, for example, Handelsman *et al.* (1991) *Appl. Environ. Microbiol.* 56:713-718). Genes encoding the pesticidal proteins of the invention can be introduced into a root-colonizing *Bacillus cereus* by standard methods known in the art.

Genes encoding pesticidal proteins can be introduced, for example, into the root-colonizing *Bacillus* by means of electrotransformation. Specifically, genes encoding the pesticidal proteins can be cloned into a shuttle vector, for example, pHT3101 (Lerecius *et al.* (1989) *FEMS Microbiol. Letts.* 60:211-218. The shuttle vector pHT3101 containing the coding sequence for the particular pesticidal protein gene can, for example, be transformed into the root-colonizing *Bacillus* by means of electroporation (Lerecius *et al.* (1989) *FEMS Microbiol. Letts.* 60:211-218).

Expression systems can be designed so that pesticidal proteins are secreted outside the cytoplasm of gram-negative bacteria, *E. coli*, for example. Advantages of having pesticidal proteins secreted are: (1) avoidance of potential cytotoxic effects of the pesticidal protein expressed, and (2) improvement in the efficiency of purification of the pesticidal protein, including, but not limited to, increased efficiency in the recovery and

purification of the protein per volume cell broth and decreased time and/or costs of recovery and purification per unit protein.

Pesticidal proteins can be made to be secreted in *E. coli*, for example, by fusing an appropriate *E. coli* signal peptide to the amino-terminal end of the pesticidal protein. Signal peptides recognized by *E. coli* can be found in proteins already known to be secreted in *E. coli*, for example the OmpA protein (Ghrayeb *et al.* (1984) *EMBO J,* 3:2437-2442). OmpA is a major protein of the *E. coli* outer membrane, and thus its signal peptide is thought to be efficient in the translocation process. Also, the OmpA signal peptide does not need to be modified before processing as may be the case for other signal peptides, for example lipoprotein signal peptide (Duffaud *et al.* (1987) *Meth. Enzymol.* 153:492).

Pesticidal proteins of the invention can be fermented in a bacterial host and the resulting bacteria processed and used as a microbial spray in the same manner that *Bacillus thuringiensis* strains have been used as insecticidal sprays. In the case of a pesticidal protein(s) that is secreted from *Bacillus*, the secretion signal is removed or mutated using procedures known in the art. Such mutations and/or deletions prevent secretion of the pesticidal protein(s) into the growth medium during the fermentation process. The pesticidal proteins are retained within the cell, and the cells are then processed to yield the encapsulated pesticidal proteins. Any suitable microorganism can be used for this purpose. *Pseudomonas* has been used to express *Bacillus thuringiensis* endotoxins as encapsulated proteins and the resulting cells processed and sprayed as an insecticide (Gaertner *et al.* (1993), *in*: Advanced Engineered Pesticides, ed. Kim).

Alternatively, the pesticidal proteins are produced by introducing a heterologous gene into a cellular host. Expression of the heterologous gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. These cells are then treated under conditions that prolong the activity of the toxin produced in the cell when the cell is applied to the environment of target pest(s). The resulting product retains the toxicity of the toxin. These naturally encapsulated pesticidal proteins may then be formulated in accordance with conventional techniques for application to the environment hosting a target pest, e.g., soil, water, and foliage of plants. See, for example EPA 0192319, and the references cited therein.

In the present invention, a transformed microorganism, which includes whole organisms, cells, spore(s), pesticidal protein(s), pesticidal component(s), pest-impacting component(s), mutant(s); preferably living or dead cells and cell components, including mixtures of living and dead cells and cell components, and including broken cells and cell components, or an isolated pesticidal protein, can be formulated with an acceptable carrier into a pesticidal composition(s) that is, for example, a suspension, a solution, an emulsion, a dusting powder, a dispersible granule, a wettable powder, and an emulsifiable concentrate, an aerosol, an impregnated granule, an adjuvant, a coatable paste, and also encapsulations in, for example, polymer substances.

Such compositions disclosed above may be obtained by the addition of a surface-active agent, an inert carrier, a preservative, a humectant, a feeding stimulant, an attractant, an encapsulating agent, a binder, an emulsifier, a dye, a UV protectant, a buffer, a flow agent or fertilizers, micronutrient donors, or other preparations that influence plant growth. One or more agrochemicals including, but not limited to, herbicides, insecticides, fungicides, bactericides, nematocides, molluscicides, acaracides, plant growth regulators, harvest aids, and fertilizers, can be combined with carriers, surfactants or adjuvants customarily employed in the art of formulation or other components to facilitate product handling and application for particular target pests. Suitable carriers and adjuvants can be solid or liquid and correspond to the substances ordinarily employed in formulation technology, e.g., natural or regenerated mineral substances, solvents, dispersants, wetting agents, tackifiers, binders, or fertilizers. The active ingredients of the present invention are normally applied in the form of compositions and can be applied to the crop area or plant to be treated, simultaneously or in succession, with other compounds. Methods of applying an active ingredient of the present invention or an agrochemical composition of the present invention that contains at least one of the pesticidal proteins produced by the bacterial strains of the present invention include, but are not limited to, foliar application, seed coating, and soil application. The number of applications and the rate of application depend on the intensity of infestation by the corresponding pest.

Suitable surface-active agents include, but are not limited to, anionic compounds such as a carboxylate of, for example, a metal; carboxylate of a long chain fatty acid; an

N-acylsarcosinate; mono or di-esters of phosphoric acid with fatty alcohol ethoxylates or salts of such esters; fatty alcohol sulfates such as sodium dodecyl sulfate, sodium octadecyl sulfate or sodium cetyl sulfate; ethoxylated fatty alcohol sulfates; ethoxylated alkylphenol sulfates; lignin sulfonates; petroleum sulfonates; alkyl aryl sulfonates such as alkyl-benzene sulfonates or lower alkylnaphtalene sulfonates, e.g., butyl-naphthalene sulfonate; salts of sulfonated naphthalene-formaldehyde condensates; salts of sulfonated phenol-formaldehyde condensates; more complex sulfonates such as the amide sulfonates, e.g., the sulfonated condensation product of oleic acid and N-methyl taurine; or the dialkyl sulfosuccinates, e.g., the sodium sulfonate or dioctyl succinate. Non-ionic agents include condensation products of fatty acid esters, fatty alcohols, fatty acid amides or fatty-alkyl- or alkenyl-substituted phenols with ethylene oxide, fatty esters of polyhydric alcohol ethers, e.g., sorbitan fatty acid esters, condensation products of such esters with ethylene oxide, e.g., polyoxyethylene sorbitar fatty acid esters, block copolymers of ethylene oxide and propylene oxide, acetylenic glycols such as 2,4,7,9-tetraethyl-5-decyn-4,7-diol, or ethoxylated acetylenic glycols. Examples of a cationic surface-active agent include, for instance, an aliphatic mono-, di, or polyamine such as an acetate, naphthenate or oleate; or oxygen-containing amine such as an amine oxide of polyoxyethylene alkylamine; an amide-linked amine prepared by the condensation of a carboxylic acid with a di- or polyamine; or a quaternary ammonium salt.

Examples of inert materials include but are not limited to inorganic minerals such as kaolin, phyllosilicates, carbonates, sulfates, phosphates, or botanical materials such as cork, powdered corncobs, peanut hulls, rice hulls, and walnut shells.

The compositions of the present invention can be in a suitable form for direct application or as a concentrate of primary composition that requires dilution with a suitable quantity of water or other diluant before application. The pesticidal concentration will vary depending upon the nature of the particular formulation, specifically, whether it is a concentrate or to be used directly. The composition contains 1 to 98% of a solid or liquid inert carrier, and 0 to 50%, preferably 0.1 to 50% of a surfactant. These compositions will be administered at the labeled rate for the commercial product, preferably about 0.01 lb-5.0 lb. per acre when in dry form and at about 0.01 pts. - 10 pts. per acre when in liquid form.

In a further embodiment, the compositions, as well as the transformed microorganisms and pesticidal proteins, of the invention can be treated prior to formulation to prolong the pesticidal activity when applied to the environment of a target pest as long as the pretreatment is not deleterious to the activity. Such treatment can be by chemical and/or physical means as long as the treatment does not deleteriously affect the properties of the composition(s). Examples of chemical reagents include but are not limited to halogenating agents; aldehydes such a formaldehyde and glutaraldehyde; anti-infectives, such as zephiran chloride; alcohols, such as isopropanol and ethanol; and histological fixatives, such as Bouin's fixative and Helly's fixative (see, for example, Humason, (1967) Animal Tissue Techniques (W.H. Freeman and Co.).

In other embodiments of the invention, it may be advantageous to treat the Cry8-like polypeptides with a protease, for example trypsin, to activate the protein prior to application of a pesticidal protein composition of the invention to the environment of the target pest. Methods for the activation of protoxin by a serine protease are well known in the art. See, for example, Cooksey (1968) Biochem. J. 6:445-454 and Carroll and Ellar (1989) Biochem. J. 261:99-105, the teachings of which are herein incorporated by reference. For example, a suitable activation protocol includes, but is not limited to, combining a polypeptide to be activated, for example a purified 1218-1 polypeptide, and trypsin at a 1/100 weight ratio 1218-1 protein/trypsin in 20nM NaHCO3, pH 8 and digesting the sample at 36° C for 3 hours.

The compositions, as well as the transformed microorganisms and pesticidal proteins, of the invention can be applied to the environment of an insect pest by, for example, spraying, atomizing, dusting, scattering, coating or pouring, introducing into or on the soil, introducing into irrigation water, by seed treatment or general application or dusting at the time when the pest has begun to appear or before the appearance of pests as a protective measure. It is generally important to obtain good control of pests in the early stages of plant growth, as this is the time when the plant can be most severely damaged. The compositions of the invention can conveniently contain another insecticide if this is thought necessary. In an embodiment of the invention, the composition is applied directly to the soil, at a time of planting, in granular form of a composition of a carrier and dead cells of a *Bacillus* strain or transformed microorganism of the invention.

Another embodiment is a granular form of a composition comprising an agrochemical such as, for example, a herbicide, an insecticide, a fertilizer, an inert carrier, and dead cells of a *Bacillus* strain or transformed microorganism of the invention.

The embodiments of the present invention may be effective against a variety of pests. For purposes of the present invention, pests include, but are not limited to, insects, fungi, bacteria, nematodes, acarids, protozoan pathogens, animal-parasitic liver flukes, and the like. Preferred pests are insect pests, particularly insect pests that cause significant damage, most particularly insect pests that cause significant damage to agricultural plants. By "insect pests" is intended insects and other similar pests such as, for example, those of the order Acari including, but not limited to, mites and ticks. Insect pests of the present invention include, but are not limited to, insects of the order Lepidoptera, e.g. *Achoroia grisella, Acleris gloverana, Acleris variana, Adoxophyes orana, Agrotis ipsilon, Alabama argillacea, Alsophila pometaria, Amyelois transitella, Anagasta kuehniella, Anarsia lineatella, Anisota senatoria, Antheraea pernyi, Anticarsia gemmatalis, Archips sp., Argyrotaenia sp., Athetis mindara, Bombyx mori, Bucculatrix thurberiella, Cadra cautella, Choristoneura sp., Cochylls hospes, Colias eurytheme, Corcyra cephalonica, Cydia latiferreanus, Cydia pomonella, Datana integerrima, Dendrolimus sibericus, Desmia feneralis, Diaphania hyalinata, Diaphania nitidalis, Diatraea grandiosella, Diatraea saccharalis, Ennomos subsignaria, Eoreuma loftini, Esphestia elutella, Erannis tilaria, Estigmene acrea, Eulia salubricola, Eupocoellia ambiguella, Eupoecilia ambiguella, Euproctis chrysorrhoea, Euxoa messoria, Galleria mellonella, Grapholita molesta, Harrisina americana, Helicoverpa subflexa, Helicoverpa zea, Heliothis virescens, Hemileuca oliviae, Homoeosoma electellum, Hyphantia cunea, Keiferia lycopersicella, Lambdina fiscellaria fiscellaria, Lambdina fiscellaria lugubrosa, Leucoma salicis, Lobesia botrana, Loxostege sticticalis, Lymantria dispar, Macalla thyrisalis, Malacosoma sp., Mamestra brassicae, Mamestra configurata, Manduca quinquemaculata, Manduca sexta, Maruca testulalis, Melanchra picta, Operophtera brumata, Orgyia sp., Ostrinia nubilalis, Paleacrita vernata, Papilio cresphontes, Pectinophora gossypiella, Phryganidia californica, Phyllonorycter blancardella, Pieris napi, Pieris rapae, Plathypena scabra, Platynota flouendana, Platynota stultana, Platyptilia carduidactyla, Plodia interpunctella, Plutella xylostella, Pontia protodice,*

Pseudaletia unipuncta, Pseudoplasia includens, Sabulodes aegrotata, Schizura concinna, Sitotroga cerealella, Spilonta ocellana, Spodoptera sp., Thaurnstopoea pityocampa, Tinsola bisselliella, Trichoplusia hi, Udea rubigalis, Xylomyges curiails, and Yponomeuta padella.

Also, the embodiments of the present invention may be effective against insect pests including insects selected from the orders Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthroptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc., particularly Coleoptera, especially *Diabrotica virgifera* and Lepidoptera. Insect pests of the invention for the major crops include: Maize: *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Helicoverpa zea*, corn earworm; *Spodoptera frugiperda*, fall armyworm; *Diatraea grandiosella*, southwestern corn borer; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Diatraea saccharalis*, surgarcane borer; *Diabrotica virgifera*, western corn rootworm; *Diabrotica longicornis barberi*, northern corn rootworm; *Diabrotica undecimpunctata howardi*, southern corn rootworm; *Melanotus spp.*, wireworms; *Cyclocephala borealis*, northern masked chafer (white grub); *Cyclocephala immaculata*, southern masked chafer (white grub); *Popillia japonica*, Japanese beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*, corn leaf aphid; *Anuraphis maidiradicis*, corn root aphid; *Blissus leucopterus leucopterus*, chinch bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Hylemya platura*, seedcorn maggot; *Agromyza parvicornis*, corn bloth leafminer; *Anaphothrips obscrurus*, grass thrips; *Solenopsis milesta*, thief ant; *Tetranychus urticae*, two spotted spider mite; Sorghum: *Chilo partellus*, sorghum borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Elasmopalpus lignosellus*, leser cornstalk borer; *Feltia subterranea*, granulate cutworm; *Phyllophaga crinita*, white grub; *Eleodes, Conoderus*, and *Aeolus spp.*, wireworms; *Oulema melanopus*, cereal leaf beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*; corn leaf aphid; *Sipha flava*, yellow sugarcane aphid; *Blissus leucopterus leucopterus*, chinch bug; *Contarinia sorghicola*, sorghum midge; *Tetranychus cinnabarinus*, carmine spider mite; *Tetranychus urticae*, two-spotted spider mite; Wheat: *Pseudaletia unipunctata*, army worm; *Spodoptera*

frugiperda, fall armyworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Agrotis orthogonia*, pale western cutworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Oulema melanopus*, cereal leaf beetle; *Hypera punctata*, clover leaf weevil; *Diabrotica undecimpunctata howardi*, southern corn rootworm; Russian wheat aphid; *Schizaphis graminum*, greenbug; *Macrosiphum avenae*, English grain aphid; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Mayetiola destructor*, Hessian fly; *Sitodiplosis mosellana*, wheat midge; *Meromyza americana*, wheat stem maggot; *Hylemya coarctata*, wheat bulb fly; *Frankliniella fusca*, tobacco thrips; *Cephus cinctus*, wheat stem sawfly; *Aceria tulipae*, wheat curl mite; Sunflower: *Cylindrocupturus adspersus*, sunflower stem weevil; *Smicronyx fulus*, red sunflower seed weevil; *Smicronyx sordidus*, gray sunflower seed weevil; *Suleima helianthana*, sunflower bud moth; *Homoeosoma electellum*, sunflower moth; *Zygogramma exclamationis*, sunflower beetle; *Bothyrus gibbosus*, carrot beetle; *Neolasioptera murtfeldtiana*, sunflower seed midge; Cotton: *Heliothis virescens*, tobacco budworm; *Helicoverpa zea*, cotton bollworm; *Spodoptera exigua*, beet armyworm; *Pectinophora gossypiella*, pink bollworm; *Anthonomus grandis grandis*, boll weevil; *Aphis gossypii*, cotton aphid; *Pseudatomoscelis seriatus*, cotton fleahopper; *Trialeurodes abutilonea*, bandedwinged whitefly; *Lygus lineolaris*, tarnished plant bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Thrips tabaci*, onion thrips; *Franklinkiella fusca*, tobacco thrips; *Tetranychus cinnabarinus*, carmine spider mite; *Tetranychus urticae*, two-spotted spider mite; Rice: *Diatraea saccharalis*, sugarcane borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Colaspis brunnea*, grape colaspis; *Lissorhoptrus oryzophilus*, rice water weevil; *Sitophilus oryzae*, rice weevil; *Nephotettix nigropictus*, rice leafhoper; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; Soybean: *Pseudoplusia includens*, soybean looper; *Anticarsia gemmatalis*, velvetbean caterpillar; *Plathypena scabra*, green cloverworm; *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Spodoptera exigua*, beet armyworm; *Heliothis virescens*, tobacco budworm; *Helicoverpa zea*, cotton bollworm; *Epilachna varivestis*, Mexican bean beetle; *Myzus persicae*, green peach aphid; *Empoasca fabae*, potato leafhopper; *Acrosternum*

hilare, green stink bug; *Melanoplus femurrubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Hylemya platura*, seedcorn maggot; *Sericothrips variabilis*, soybean thrips; *Thrips tabaci*, onion thrips; *Tetranychus turkestani*, strawberry spider mite; *Tetranychus urticae*, two-spotted spider mite; Barley: *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; *Schizaphis graminum*, greenbug; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; *Euschistus servus*, brown stink bug; *Jylemya platura*, seedcorn maggot; *Mayetiola destructor*, Hessian fly; *Petrobia latens*, brown wheat mite; Oil Seed Rape: *Vrevicoryne brassicae*, cabbage aphid; *Phyllotreta cruciferae*, crucifer flea beetle; Potato: *Leptinotarsa decemlineata,* Colorado potato beetle.

Furthermore, embodiments of the present invention may be effective against *Hemiptera* such *as Lygus hesperus, Lygus lineolaris, Lygus pratensis, Lygus rugulipennis Popp, Lygus pabulinus, Calocoris norvegicus, Orthops compestris, Plesiocoris rugicollis, Cyrtopeltis modestus, Cyrtopeltis notatus, Spanagonicus albofasciatus, Diaphnocoris chlorinonis, Labopidicola allii, Pseudatomoscelis seriatus, Adelphocoris rapidus, Poecilocapsus lineatus, Blissus leucopterus, Nysius ericae, Nysiusraphanus, Euschistus servus, Nezara viridula, Eurygaster, Coreidae, Pyrrhocoridae, Tinidae, Blostomatidae, Reduviidae,* and *Cimicidae.*

Nematodes include plant-parasitic nematodes such as root-knot, cyst, and lesion nematodes, including *Heterodera* and *Globodera spp;* particularly *Globodera rostochiensis* and *Globodera pailida* (potato cyst nematodes); *Heterodera glycines* (soybean cyst nematode); *Heterodera schachtii* (beet cyst nematode); and *Heterodera avenae* (cereal cyst nematode).

The preferred developmental stage for testing for pesticidal activity is larvae or immature forms of these above mentioned insect pests. The insects may be reared in total darkness at from about 20°C to about 30°C and from about 30% to about 70% relative humidity. Bioassays may be performed as described in Czapla and Lang (1990) *J. Econ. Entomol.* 83(6): 2480-2485. Methods of rearing insect larvae and performing bioassays are well known to one of ordinary skill in the art.

A wide variety of bioassay techniques is known to one skilled in the art. General procedures include addition of the experimental compound or organism to the diet source

in an enclosed container. Pesticidal activity can be measured by, but is not limited to, mortality, weight loss, attraction, repellency and other behavioral and physical changes after feeding and exposure for an appropriate length of time. Bioassays described herein can be used with any feeding insect pest in the larval or adult stage.

The following examples are presented by way of illustration, not by way of limitation.

EXPERIMENTAL

Example 1: Bioassay for Testing the Pesticidal Activity of *B. thuringiensis* Strains Against Western Corn Rootworm and Southern Corn Rootworm

Insect diets for Colorado potato beetle (CPB), southern corn rootworm (SCRW), and western corn rootworm (WCRW) larvae are known in the art. See, for example, Rose and McCabe (1973) *J. Econ. Entomology* 66:393, herein incorporated by reference. The insect diet is prepared and poured onto a Pittman tray. Generally 1.5 mL of diet is dispensed into each cell with an additional 150 µL of sample preparation applied to the diet surface.

Bacterial colonies from an original plate of transformants expressing the pesticidal proteins of interest are spotted on replica plates and inoculated in 5 mL 2x YT broth with 500 µL/1000 mL kanamycin antibiotic. The tubes are grown overnight. If no growth is present, the tubes are incubated for an additional 24 hours. Following incubation, the tubes are centrifuged at 3500 rpms for 5-8 minutes. The supernatant is discarded and the pellet resuspended in 1000 µL PBS. The sample is then transferred to 1.5 mL eppendorf tubes and incubated on ice until the temperature is 3 to 4°C, followed by sonication for 12-15 seconds.

Microbial culture broths (150 µL) or other samples (150 µL) are overlayed onto 1.5 mL artificial diets with a 2.54 cm^2 surface area. For the screening of pesticidal activity against rootworms, 25 µL of a 0.8% egg agar solution is applied to lids of the trays. The trays and lids are allowed to dry under a hood. After drying, the lids are placed on trays and incubated for 4-7 days at a temperature of 26°C. The bioassays are then scored by counting "live" versus "dead" larvae. Mortality is calculated as percentage of dead larvae out of the total larvae tested.

Example 2: Pesticidal Activity of *B. thuringiensis* strain 1218 Lysates

Samples prepared from cultures of *B. thuringiensis* strains 1218 were tested for the presence of pesticidal activity against CPB, WCRW, and SCRW as described in Example 1. As a control, the diet was treated with phosphate-buffered saline (PBS).

To prepare each sample, an individual colony of a strain growing on an LB plate was selected and used to inoculate a tube containing 50 mL of TB medium. The tube was incubated overnight at 28°C and 250 rpm. Following the incubation, the tube was centrifuged at 4300 x *g* for 15 minutes. The supernatant was discarded and the pellet resuspended in 50 mL of sporulation medium. The tube was centrifuged again at 4300 x *g* for 15 minutes. The second supernatant was discarded, and the second pellet resuspended in 50 mL of sporulation medium. The tube was then incubated for 48 hours at 28°C and 250 rpm. Following this incubation, the tube was centrifuged at 4300 x *g* for 15 minutes. The supernatant was discarded, and the pellet was resuspended in 10 mL of 1X M9 medium. The sample was then transferred to a 1.5 mL microfuge tube, incubated on ice until the temperature was about 3 to 4°C, and then sonicated for 12-15 seconds. For bioassays, 150 µL of a sonicated sample was used.

Sporulation medium comprises 200 mL of 5X M9 salts solution, 5 mL of salts solution, 5 mL of $CaCl_2$ solution, and dH_20 to a final volume of 1 L. The solution of 5X M9 salts comprises: 64 g, $Na_2HPO_4 \cdot 7H_2O$; 15 g, KH_2PO_4; 2.5 g, NaCl; 5 g, NH_4Cl; and dH2O to a final volume of 1.0 L. Salts solution comprises: 2.46 g, $MgSO_4 \cdot 7H_2O$; 0.04 g, $MnSO_4 \cdot H2O$; 0.28 g, $ZnSO_4 \cdot 7H2O$; 0.40 g, $FeSO_4 \cdot 7H2O$; and dH_2O to a final volume of 1.0 L. $CaCl_2$ solution comprises 3.66 g $CaCl_2 \cdot 2H_2O$ and dH2O to a final volume of 100 ml.

Samples were tested with and without heating to determine whether the component(s) responsible for the pesticidal activity is heat stable. For the heat treatment, the samples were boiled for 15 minutes prior to use in the bioassay. Unheated samples prepared from strain 1218 exhibited pesticidal activity against western corn rootworm, with lesser pesticidal activity against southern corn rootworm. The samples prepared from strain 1218 lysates caused moderate stunting in the southern corn rootworm larvae. Following heating, the samples had greatly reduced pesticidal activity against both species of rootworms.

The reduction in pesticidal activity following heating indicated that the one or more components of the sample from strain 1218 that is responsible for the pesticidal activity is heat labile. Such a reduction is consistent with one or more of the components being a protein.

Example 3: Pesticidal Activity of Crystal Proteins Isolated from *B. thuringiensis* Strain 1218

Using samples of sporulated cultures of *B. thuringiensis* strain 1218 prepared as described in Example 2, crystal proteins were isolated and then trypsin-treated using methods known in the art. Briefly, after purification (zonal gradient centrifugation, Renografin-76), the purified crystals were dissolved in alkaline buffer (50 mM Na_2CO_3, 10 mM dithiothreitol, pH 10). Prior to use in the assays, the dissolved crystal proteins were concentrated by filtration with Centriprep® (Millipore Corp.) centrifugal filter units with a MW cutoff of 10,000.

It is recognized that under some experimental conditions, it may be advantageous to treat the Cry8-like polypeptides with a protease, for example trypsin, to activate the protein prior to determining the pesticidal activity of a particular sample. Methods for the activation of protoxin by a serine protease are well known in the art. See, for example, Cooksey (1968) *Biochem J.* 6:445-454 and Carroll and Ellar (1989) *Biochem J.* 261:99-105; herein incorporated by reference. Isolated crystal proteins were screened for pesticidal activity against western corn rootworm larvae as described in Example 1. Both a new crystal protein preparation and a previously made preparation ("old preparation")

from strain 1218 possessed substantial pesticidal activity against western corn rootworms. Dissolved crystal proteins were stored at –80° C for 20 days before use in the assays.

A skilled artisan will acknowledge that there are numerous indicators of pesticidal activity and that variables such as number of dead insects, or average weight of treated insects can be monitored. For example, pesticidal activity can be conveniently expressed as % mortality, which is the percentage of dead rootworm larvae out of the total number of larvae.

Example 4: Nucleotide Sequences Isolated from *B. thuringiensis* Strain 1218

An effort was undertaken to isolate the nucleotide sequences that encode the crystal proteins from *B. thuringiensis* strain 1218. Two nucleotide sequences were isolated from 1218 that have nucleotide sequence and amino acid sequence homology to *Cry8Ba1* (GenBank Accession No. U04365). The two *Cry8*-like coding sequences isolated from strain 1218 have been designated *Cry1218-1* (SEQ ID NO:1) and *Cry1218-2* (SEQ ID NO:3). SEQ ID NO:27 and SEQ ID NO:28 provide the nucleic acid sequences of native genomic clones of *Cry1218-1* and *Cry1218-2*, respectively.

To determine if the proteins encoded by variant or mutant polynucleotides of the invention encode proteins with pesticidal activity, each of the nucleic acid sequence was expressed in *Escherichia coli*. For example, to determine if the 1218-1 or 1218-2 polynucleotide sequences provided herein encode polypeptides with pesticidal activity, truncated nucleotide sequences were prepared. SEQ ID NO:15 corresponds to nucleotides 1 through 2007 of the nucleotide sequence of *Cry1218-1* (SEQ ID NO:1). SEQ ID NO:17 corresponds to nucleotides 1 through 2019 of the nucleotide sequence of *Cry1218-2* (SEQ ID NO:3).

SEQ ID NOS:15 and 17 encode truncated Cry8-like polypeptides having the amino acid sequences set forth in SEQ ID NO:16 and 18, respectively. Each of the truncated nucleotide sequences (SEQ ID NOs:15 and 17) was separately cloned into a pET28a expression vector and then used to transform *E. coli*. Transformed colonies were selected and grown in liquid culture as described in Example 1. The expressed, N-terminal-His-tagged, truncated Cry8-like proteins were isolated from *E. coli* lysates by

affinity chromatography using a Nickel affinity column. The column fractions with the protein of interest were dialyzed extensively against 10 mM Tris-HCl (pH 8.5) and then concentrated using Centriprep® (Millipore Corp.) centrifugal filter units with a MW cutoff of 10,000 according to the manufacturer's directions. The concentrated Cry8-like protein samples were tested for the presence of pesticidal activity against western corn rootworm as described in Example 1.

Bioassays evaluating the pesticidal activity of recombinant Cry8-like proteins purified from *E. coli*-expressed preparations were conducted as described in Example 1 with the aqueous protein samples overlaid on the surface of the rootworm diet. The pesticidal activity of wild-type (e.g., native) and mutant endotoxin were assessed against southern corn rootworms. As expected, it was observed that the pesticidal activity decreased as the concentration of the truncated Cry8-like proteins applied to the diet decreased.

Pesticidal activity was also assessed by incorporating the pesticidal proteins into the rootworm diet, as opposed to the method described above, which involved incorporating a protein-containing solution into the diet mixture. For example, sample diets comprising 1000, 500, 400, 300, 200, or 100 ppm of a pesticidal polypeptide incorporated into the diet were assessed.

Example 5: Preparation of a Plant-Preferred Nucleotide Sequence Encoding a Pesticidal Protein

Because codon usage is different between plants and bacteria, the expression in a plant of a protein encoded by nucleotide sequence of bacterial origin can be limited due to translational inefficiency in the plant. It is known in the art that expression can be increased in a plant by altering the coding sequence of the protein to contain plant-preferred codons. For optimal expression of a protein in a plant, a synthetic nucleotide sequence may be prepared using the amino acid sequence of the protein and back-translating the sequence using plant-preferred codons.

Using such an approach, a portion of the amino acid sequence of the protein encoded by *Cry1218-1* (SEQ ID NO:2) was back-translated using maize-preferred codons. The resulting plant-preferred nucleotide sequence is set forth in SEQ ID NO:9.

The nucleotide sequence set forth in SEQ ID NO:9 encodes a polypeptide (SEQ ID NO:10) that comprises the first 669 amino acids of the amino acid sequence set forth in SEQ ID NO:2. Thus, SEQ ID NOS:10 and 16 encode polypeptides comprising the same amino acid sequence, and SEQ ID NO:15 provides a second polynucleotide that encodes the amino acid sequences set forth in SEQ ID NO:10.

Example 6: Bioassay for Testing the Pesticidal Activity of Mutant Cry8-like Polypeptides against Colorado Potato Beetle *(Leptinotarsa decemlineata)*

Protocol

Briefly, bioassay parameters were as follows: Bio-Serv diet (catalog number F9800B, from: BIOSERV, Entomology Division, One 8th Street , Suite 1, Frenchtown, New Jersey 08825) was dispensed in 128-well Pitman trays (catalog number BIO-BA-128 from CD International, Pitman, New Jersey 08071) having a surface area of 2.4 cm^2. Cry 8-like samples (1218-1A, 49PVD, and NGSR1218-1) were applied topically to the diet surface at a rate of 50 µl/well. Enough sample material was supplied to provide for 4 observations/sample. After the sample dried, 2 Colorado potato beetle neonates were added to each well. Therefore, there was a total of 8 larvae/sample. A lid was placed on each tray (catalog number BIO-CV-16, CD International, Pitman, New Jersey, 08071) and the trays were placed in an incubator at 25°C.

The assay trays showed no surface contamination present in the buffer controls or the wells that contained Cry8-like samples. The test was scored for mortality on the 4th day following live infesting.

TABLE 1. Pesticidal Activity of Truncated 1218-1 Polypeptides and a Trypsin Addition-Mutant against Colorado Potato Beetle

Code	Samples	Protein (mg/ml)	Mortality	
A	a-buffer		1/8	13%
B	b-1218-1A	0.05	7/8	88%
C	c-1218-1A	0.025	7/8	88%
D	d-1218-1A	0.013	4/6	67%
F	f-49PVD	0.1	8/8	100%
G	g-49PVD	0.05	4/8	50%
H	h-49PVD	0.025	8/9	89%
L	l-NGSR1218-1	0.1	8/8	100%
M	m-NGSR1218-1	0.05	8/8	100%
N	n-NGSR1218-1	0.025	8/8	100%

Results

The sample labeled "A" in Table 1 is a control sample consisting of 10 mM carbonate buffer at pH 10. All of the truncated and mutant protein samples 1218-1A (b-d), 49PVD (f-h), and NGSR1218-1 (l-n) were solubilized in 10 mM carbonate buffer at pH 10.

The 1218-1A samples, b-d, comprise a truncated polypeptide sequence comprising the amino acid sequence set forth in SEQ ID NO:16. More specifically, the 1218-1A samples comprise the truncated toxin domain represented by amino acid (aa) residue 1 to aa 669 (from M to E) of the amino acid sequences set forth in SEQ ID NO:2.

The 49PVD samples, f-h, comprise a mutant polypeptide sequence having an amino acid sequence that is set forth in SEQ ID NO:20. 49PVD was generated by trimming sequence from both the N-terminus and the C-terminus of the sequence set forth in SEQ ID NO:16. More specifically, the N-terminus of the 49PVD mutant was trimmed by 47 residues; thus, the polypeptide starts at aa residue 48(M) and the C-

1218-1A (SEQ ID NO:16) from aa residue 48 to aa 663.

The NGSR samples, l-m, comprise a 1218-1 mutant polypeptide sequence that is set forth in SEQ ID NO:12. NGSR1218-1 was generated by the addition of an NGSR motif to the amino acid sequence set forth in SEQ ID NO:16 after aa 164. More specifically, the NGSR mutant provides a 1218-1A mutant that includes the amino acid sequence NGSR between aa 164 and aa 165 of the sequence set forth in SEQ ID NO:16. The addition of 4 residues to 1218-1A generated a protein with 673 aa. Bioassays of 1218-1A, 49PVD, and NGSR1218-1 indicated that all three protein samples are efficacious against Colorado potato beetle (CPB). Mutant NGSR1218-1 was found to be more potent that the parent 1218-1A and 49PVD mutant. The modified (e.g., truncated or mutant) 1218-1 polypeptides (49PVD, NGSR1218-1) were at least as active as the relevant 1218-1 or 1218-1A control sample.

Example 7: Bioassay for Testing the Pesticidal Activity of Mutant Cry8-like Polypeptides against Southern Corn Rootworm and Western Corn Rootworm

Protocol

Briefly, the assay parameters described above in Example 6 were modified to allow for the evaluation of the pesticidal activity of additional 1218-1,1218-1A or 49PVD mutants against western corn rootworm (WCRW) and southern corn rootworm (SCRW). Briefly, Bio-Serv diet (catalog number F9800B, from: BIOSERV, Entomology Division, One 8th Street, Suite 1, Frenchtown, New Jersey 08825) was dispensed in 128-well Pitman trays (catalog number BIO-BA-128 from CD International, Pitman, New Jersey 08071) having a surface area of 2.4 cm^2.

Cry 8-like samples were applied topically to the diet surface at a volume of 50 µl/well. Enough sample material was supplied to provide for replicate observations/sample. For the screening of pesticidal activity against rootworms, 25 µL of a 0.8% egg agar solution is applied to lids of the trays. The trays and lids are allowed to dry under a hood. After drying, the lids are placed on trays and incubated for 4-7 days at a temperature of 26°C. A lid was placed on each tray (catalog number BIO-CV-16, CD

International, Pitman, New Jersey, 08071), and the trays were placed in an incubator at 25°C.

For the evaluation of pesticidal activity against SCRW, insects were exposed to a solution comprising either buffer (50 mM carbonate buffer (pH 10) or a 1218-1 or 1218-1A mutant polypeptide (e.g., 1218-1A), LKMS.N1218-1, LKMS.R1218-1, NGSR.N1218-1, LKMS.N49PVD, LKMS.R49PVD, or NGSR.N49PVD) at a doses of either 36 or 3.6 $\mu g/cm^2$.

For the evaluation of pesticidal activity against WCRW, insects were exposed to a solution comprising either buffer (50 mM carbonate buffer (pH 10) or to a limited number of the mutant 1218-1 polypeptides (LKMS.R1218-1, NGSR.N1218-1, LKMS.N49PVD, LKMS.R49PVD, or NGSR.N49PVD) at 88 $\mu g/cm^2$.
The bioassays are then scored by counting "live" versus "dead" larvae. Mortality is calculated as percentage of dead larvae out of the total larvae tested.

TABLE 2. Pesticidal Activity of *Cry1218-1* Mutant Polypeptides against Southern Corn Rootworm- Replicate 1

POLYPEPTIDE	DOSE	% MORTALITY	DOSE	% MORTALITY
1218-1A	36 $\mu g/cm^2$	0	3.6 $\mu g/cm^2$	3
LKMS.N 1218-1	36 $\mu g/cm^2$	6 (no protein)	3.6 $\mu g/cm^2$	4
LKMS.R 1218-1	36 $\mu g/cm^2$	89	3.6 $\mu g/cm^2$	27
NGSR.N 1218-1	36 $\mu g/cm^2$	80	3.6 $\mu g/cm^2$	8
50 mM Carbonate Buffer (pH10)	-	0	-	0
49PVD	36 $\mu g/cm^2$	3	3.6 $\mu g/cm^2$	3
LKMS.N49PVD	36 $\mu g/cm^2$	69	3.6 $\mu g/cm^2$	11
LKMS.R49PVD	36 $\mu g/cm^2$	60	3.6 $\mu g/cm^2$	17
NGSR.N49PVD	36 $\mu g/cm^2$	93	3.6 $\mu g/cm^2$	22

TABLE 3. Pesticidal Activity of Cry1218-1 Mutant Polypeptides against Southern Corn Rootworm- Replicate 2

POLYPEPTIDE	DOSE	% MORTALITY	DOSE	% MORTALITY
1218-1A	36 µg/cm^2	3	3.6 µg/cm^2	0
LKMS.N 1218-1				
LKMS.R 1218-1	36 µg/cm^2	75	3.6 µg/cm^2	20
NGSR.N 1218-1	36 µg/cm^2	77	3.6 µg/cm^2	23
50 mM Carbonate Buffer (pH10)	-	0	-	0
49PVD	36 µg/cm^2	0	3.6 µg/cm^2	2
LKMS.N49PVD	36 µg/cm^2	83	3.6 µg/cm^2	0
LKMS.R49PVD	36 µg/cm^2	62	3.6 µg/cm^2	3
NGSR.N49PVD	36 µg/cm^2	81	3.6 µg/cm^2	25

TABLE 4. Pesticidal Activity of Cry1218-1 Mutant Polypeptides against Southern Corn Rootworm- Replicate 3

POLYPEPTIDE	DOSE	% MORTALITY	DOSE	% MORTALITY
1218-1A	36 µg/cm^2	2	3.6 µg/cm^2	0
LKMS.N 1218-1	-	-	-	-
LKMS.R 1218-1	36 µg/cm^2	74	3.6 µg/cm^2	15
NGSR.N 1218-1	36 µg/cm^2	65	3.6 µg/cm^2	17
50 mM Carbonate Buffer (pH 10)	-	0	-	0
49PVD	36 µg/cm^2	0	3.6 µg/cm^2	0
LKMS.N49PVD	36 µg/cm^2	70	3.6 µg/cm^2	5
LKMS.R49PVD	36 µg/cm^2	57	3.6 µg/cm^2	4
NGSR.N49PVD	36 µg/cm^2	81	3.6 µg/cm^2	28

TABLE 5. Pesticidal Activity of Cry1218-1 Mutant Polypeptides against Western Corn Rootworm

POLYPEPTIDE	DOSE	% MORTALITY
1218-1A		
LKMS.N 1218-1		
LKMS.R 1218-1	88 µg/cm^2	16
NGSR.N 1218-1	88 µg/cm^2	14
50 mM Carbonate Buffer (pH 10)	-	4
49PVD	88 µg/cm^2	
LKMS.N49PVD	88 µg/cm^2	7
LKMS.R49PVD	88 µg/cm^2	12
NGSR.N49PVD	88 µg/cm^2	10

TABLE 6. Pesticidal Activity of Cry1218-1 Mutant Polypeptides against Western Corn Rootwoom

POLYPEPTIDE	DOSE	AVERAGE LARVAE WT (µg)			NUMBER OF LARVAE WEIGHED			% MORTALITY		
	($\mu g/cm^2$)	TRAY 1	TRAY 2	AVERAGE	TRAY 1	TRAY 2	AVERAGE	TRAY 1	TRAY 2	AVERAGE
1218-1A	193	161	140	150.5	74	65	69.5	9	9	9
NGSR.N 1218-1	193	92	83	99	60	60	60	24	12	18
LKMS.R 1218-1	193	92	106	99	48	49	48.5	20	13	16.5
49PVD	220	129	166	147.5	79	71	75	6	2	4
NGSR.N 49PVD	220	67	76	71.5	39	58	48.5	22	7	14.5
LKMS.R 49PVD	220	92	94	93	49	32	40.5	20	17	18.5
LKMS.N 49PVD	220	82	80	81	44	41	42.5	28	16	22
50Mm Carbonate	220	165	164	164.5	80	95	87.5	4	0	2
Diet	220	171	132	151.5	78	78	78	1	6	3.5

Example 8: Transformation of Maize by Particle Bombardment and Regeneration of Transgenic Plants

Immature maize embryos from greenhouse donor plants are bombarded with a plasmid containing the plant-optimized C*ry1218-1* nucleotide sequence (SEQ ID NO:9) operably linked to a ubiquitin promoter and the selectable marker gene PAT (Wohlleben *et al.* (1988) *Gene 70*:25-37), which confers resistance to the herbicide Bialaphos. Alternatively, the selectable marker gene is provided on a separate plasmid. Transformation is performed as follows. Media recipes follow below.

Preparation of Target Tissue

The ears are husked and surface sterilized in 30% Clorox bleach plus 0.5% Micro detergent for 20 minutes, and rinsed two times with sterile water. The immature embryos are excised and placed embryo axis side down (scutellum side up), 25 embryos per plate, on 560Y medium for 4 hours and then aligned within the 2.5-cm target zone in preparation for bombardment.

Preparation of DNA

A plasmid vector comprising the plant-optimized C*ry1218-1* nucleotide sequence (SEQ ID NO:9) operably linked to a ubiquitin promoter is made. For example, a suitable transformation vector comprises a UBI1 promoter from *Zea mays,* a 5' UTR from UBI1 and a UBI1 intron, in combination with a PinII terminator. A plasmid DNA comprising the plant-optimized nucleotide sequence and a second plasmid DNA containing a PAT selectable marker (e.g., CAMV35S(ACK) promoter driving PAT with a CAMV35S terminator) is precipitated onto 1.1 μm (average diameter) tungsten pellets using a $CaCl_2$ precipitation procedure as follows:

100 μl prepared tungsten particles in water

10 μl (1 μg) DNA in Tris EDTA buffer (1 μg total DNA)

100 μl 2.5 M $CaCl_2$

10 μl 0.1 M spermidine

Each reagent is added sequentially to a tungsten particle suspension, while maintained on the multitube vortexer. The final mixture is sonicated briefly and allowed to incubate under constant vortexing for 10 minutes. After the precipitation period, the tubes are centrifuged briefly, liquid removed, washed with 500 ml 100% ethanol, and centrifuged for 30 seconds. Again the liquid is removed, and 105 µl 100% ethanol is added to the final tungsten particle pellet. For particle gun bombardment, the tungsten/DNA particles are briefly sonicated and 10 µl spotted onto the center of each macrocarrier and allowed to dry about 2 minutes before bombardment.

Particle Gun Treatment

The sample plates are bombarded at level #4 in particle gun #HE34-1 or #HE34-2. All samples receive a single shot at 650 PSI, with a total of ten aliquots taken from each tube of prepared particles/DNA.

Subsequent Treatment

Following bombardment, the embryos are kept on 560Y medium for 2 days, then transferred to 560R selection medium containing 3 mg/liter Bialaphos, and subcultured every 2 weeks. After approximately 10 weeks of selection, selection-resistant callus clones are transferred to 288J medium to initiate plant regeneration. Following somatic embryo maturation (2-4 weeks), well-developed somatic embryos are transferred to medium for germination and transferred to the lighted culture room. Approximately 7-10 days later, developing plantlets are transferred to 272V hormone-free medium in tubes for 7-10 days until plantlets are well established. Plants are then transferred to inserts in flats (equivalent to 2.5' pot) containing potting soil and grown for 1 week in a growth chamber, subsequently grown an additional 1-2 weeks in the greenhouse, then transferred to classic 600 pots (1.6 gallon) and grown to maturity. Plants are monitored and scored for expression of the Cry1218-1 protein by assays known in the art, such as, for example, immunoassays and western blotting with an antibody that binds to the Cry1218-1 protein.

Bombardment and Culture Media

Bombardment medium (560Y) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 120.0 g/l sucrose, 1.0 mg/l 2,4-D, and 2.88 g/l L-proline (brought to volume with D-I H_20 following adjustment to pH 5.8 with KOH); 2.0 g/l Gelrite (added after bringing to volume with D-I H_20); and 8.5 mg/l silver nitrate (added after sterilizing the medium and cooling to room temperature). Selection medium (560R) comprises 4.0 g/l N6 basal salts (SIGMA C-1416), 1.0 ml/l Eriksson's Vitamin Mix (1000X SIGMA-1511), 0.5 mg/l thiamine HCl, 30.0 g/l sucrose, and 2.0 mg/l 2,4-D (brought to volume with D-I H_20 following adjustment to pH 5.8 with KOH); 3.0 g/l Gelrite (added after bringing to volume with D-I H_20); and 0.85 mg/l silver nitrate and 3.0 mg/l Bialaphos (both added after sterilizing the medium and cooling to room temperature).

Plant regeneration medium (288J) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g nicotinic acid, 0.02 g/l thiamine HCl, 0.10 g/l pyridoxine HCl, and 0.40 g/l Glycine brought to volume with polished D-I H_20) (Murashige and Skoog (1962) *Physiol. Plant. 15*:473), 100 mg/l myo-inositol, 0.5 mg/l zeatin, 60 g/l sucrose, and 1.0 ml/l of 0.1 mM abscisic acid (brought to volume with polished D-I H_20 after adjusting to pH 5.6); 3.0 g/l Gelrite (added after bringing to volume with D-I H_20); and 1.0 mg/l indoleacetic acid and 3.0 mg/l Bialaphos (added after sterilizing the medium and cooling to 60°C). Hormone-free medium (272V) comprises 4.3 g/l MS salts (GIBCO 11117-074), 5.0 ml/l MS vitamins stock solution (0.100 g/l nicotinic acid, 0.02 g/l thiamine HCl, 0.10 g/l pyridoxine HCl, and 0.40 g/l Glycine brought to volume with polished D-I H_20), 0.1 g/l myo-inositol, and 40.0 g/l sucrose (brought to volume with polished D-I H_20 after adjusting pH to 5.6); and 6 g/l bacto-agar (added after bringing to volume with polished D-I H_20), sterilized and cooled to 60° C.

Example 9: Agrobacterium-Mediated Transformation of Maize and Regeneration of Transgenic Plants

For *Agrobacterium*-mediated transformation of maize with a plant-optimized *Cry1218-1* nucleotide sequence (SEQ ID NO: 9), preferably the method of Zhao is employed (U.S. Patent No. 5,981,840, and PCT patent publication WO98/32326; the

contents of which are hereby incorporated by reference). Briefly, immature embryos are isolated from maize and the embryos contacted with a suspension of *Agrobacterium*, under conditions whereby the bacteria are capable of transferring the plant-optimized *Cry1218-1* nucleotide sequence (SEQ ID NO:9) to at least one cell of at least one of the immature embryos (step 1: the infection step). In this step the immature embryos are preferably immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos are co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). Preferably the immature embryos are cultured on solid medium following the infection step. Following this co-cultivation period an optional "resting" step is contemplated. In this resting step, the embryos are incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). Preferably the immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos are cultured on medium containing a selective agent and growing transformed callus is recovered (step 4: the selection step). Preferably, the immature embryos are cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus is then regenerated into plants (step 5: the regeneration step), and preferably calli grown on selective medium are cultured on solid medium to regenerate the plants.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended embodiments.